BRICHOS
- a Novel Anti-Amyloid Chaperone

Studies on Pro-Surfactant Protein C

Hanna Willander
Faculty of Veterinary Medicine and Animal Science
Department of Anatomy, Physiology and Biochemistry
Uppsala

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2011

Acta Universitatis agriculturae Sueciae
2011:28

Cover illustration: *Upper left:* Crystal structure of a proSP-C BRICHOS monomer with a central five stranded β-sheet with two α-helices on either side. *Upper right:* An amyloid deposit, labeled with an antibody against mature SP-C, visualized with 2,2'-diamino benzidine (brown) and then stained with Congo red and examined in polarized light (photo: Per Westermark). *Lower left:* Transmission electron micrograph of fibrils from a synthetic peptide corresponding to proSP-C residues 24-45(photo: Erik Hermansson). *Lower right*: The linker region and parts of the TM region form a β-hairpin structure that binds to face A in the BRICHOS domain.

ISSN 1652-6880
ISBN 978-91-576-7563-7

Print: SLU Service/Repro, Uppsala 2011

BRICHOS – a novel anti-amyloid chaperone. Studies on pro-surfactant protein c.

Abstract

Lung surfactant protein C (SP-C) is a 35-residue, transmembrane (TM) peptide that is extremely hydrophobic and lacks known homologous proteins. Due to a high content in Val residues in the α-helical TM part, SP-C can spontaneously convert into β-sheet aggregates. We show here that SP-C forms amyloid in interstitial lung disease (ILD) caused by mutations in the C-terminal part of proSP-C (CTC).

CTC has been predicted to contain a ~100-residue BRICHOS domain, found in 12 protein families with a wide range of functions and disease associations, such as respiratory distress syndrome, dementia and cancer. We hypothesised that the BRICHOS domain can act as a chaperone, preventing proproteins from misfolding during biosynthesis. Recombinant CTC can bind to lipid associated non-helical SP-C and this interaction results in an increased α-helical content in the mature SP-C peptide. Wildtype CTC can also stabilize proSP-C(1-58), which lacks the BRICHOS domain, and a proSP-C mutant in HEK293 cells. CTC binds selectively peptides derived from the TM part of SP-C and to residues that promote membrane insertion.

CTC can also bind to other hydrophobic peptides, in particular the amyloid β-peptide (Ab) associated with Alzheimer disease. CTC and Bri2 (associated with familial dementia) BRICHOS can prevent fibril formation of $A\beta_{40}$ and $A\beta_{42}$ far below stoichiometric amounts, indicating that BRICHOS may be useful in future therapy.

The crystal structure of the BRICHOS domain from CTC shows a novel fold with a central β-sheet flanked by α-helices. Many of the hydrophobic residues in the β-sheet are conserved and many of the point mutations associated with ILD coincide with these residues, suggesting that they are involved in the function of the BRICHOS domain, possibly by binding substrate peptides.

Taken together, results in this thesis, suggest that BRICHOS is a novel anti-amyloid chaperone domain and mutations that lead to BRICHOS dysfunction cause ILD and amyloid disease.

Keywords: BRICHOS, surfactant protein C, interstitial lung disease, chaperone, amyloid, mutation, amyloid β-peptide, crystal structure, Bri2.

Author's address: Hanna Willander, SLU, Department of Anatomy, Physiology and Biochemistry, P.O. Box 575, SE-751 23 Uppsala, Sweden
E-mail: Hanna.Willander@slu.se

Dedication

Till minnet av min farmor

Contents

List of Publications

This thesis is based on the following papers, referred to by Roman numerals (I-VI) in the text:

I **Johansson H**[1], Nordling K, Weaver TE, Johansson J. (2006). The Brichos domain-containing C-terminal part of pro-surfactant protein C binds to an unfolded poly-val transmembrane segment. *J Biol Chem.* 281(30), 21032-9.

II **Johansson H**[1], Eriksson M, Nordling K, Presto J, Johansson J. (2009). The Brichos domain of prosurfactant protein C can hold and fold a transmembrane segment. *Protein Sci.* 18(6), 1175-82.

III Casals C, **Johansson H**[1], Saenz A, Gustafsson M, Alfonso C, Nordling K, Johansson J. (2008). C-terminal, endoplasmic reticulum-lumenal domain of prosurfactant protein C – structural features and membrane interactions. *FEBS J.* 275(3), 536-47.

IV **Willander H**⋆, Askarieh G⋆, Landreh M, Westermark P, Nordling K, Keränen H, Hermansson E, Hamvas A, Nogee, LM, Bergman T, Saenz A, Casals C, Åqvist J, Jörnvall H, Berglund H, Presto J, Knight SD, Johansson J. (2011) New amyloid disease caused by aberrant chaperone activity. (manuscript). ⋆These authors contributed equally to this work.

[1] Hanna Willander former Johansson

V **Johansson H**[1]*, Nerelius C*, Nordling K, Johansson J. (2009). Preventing amyloid formation by catching unfolded transmembrane segments. *J Mol Biol.* 389(2), 227-9. *These authors contributed equally to this work.

VI **Willander H**, Presto J, Askarieh G, Frohm B, Knight S, Johansson J, Linse S. BRICHOS domains prevent fibrillation of amyloid β-peptide through destabilization of oligomeric intermediates. (manuscript)

Papers not included in the thesis:

Johansson J, Nerelius C, **Willander H**, Presto J. (2010) Conformational preferences of non-polar amino acid residues: an additional factor in amyloid formation. *Biochem Biophys Res Commun.* 402(3), 515-8.

Yang L, Johansson J, Risdale R, **Willander H**, Fitzen M, Akinbi HT, Weaver TE. (2010) Surfactant protein B contains a saposin-like protein domain with antimicrobial activity at low pH. *J Immunol.* 184(2), 975-83.

Abbreviations

Three and one letter codes for the 20 naturally occurring amino acids

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Other abbreviations used

Aβ	Amyloid β-peptide
AD	Alzheimer's disease
APP	Amyloid-β precursor protein
AUC	Analytical ultra centrifugation
Bis-ANS	1,1'-bis(4-anilino-5,5'-naphtahalenesulfonate)
CD	Circular dichroism
CTC	C-terminal part of proSP-C
DPPC	Dipalmitoyl-phosphatidylcholine
DSC	Differential scanning calorimetry
ER	Endoplasmic reticulum
ESI-MS	Electrospray ionisation mass spectrometry
FBD	Familial British dementia
FDD	Familial Danish dementia
GKN1	Gastrokine 1
GKN2	Gastrokine 2
HEK cells	Human embryonic kidney cells
HSP	Heat shock proteins
HDX	Hydrogen deuterium exchange
ITM2A	Integral transmembrane protein 2A
ITM2B	Integral transmembrane protein 2B
ITM2C	Integral transmembrane protein 2C
kDa	kilo Dalton
LB	Lamellar body
LECT1	Leukocyte cell-derived chemotaxin 1(Chondromodulin)
MALDI	Matrix assisted laser desorption ionization
MVB	Multivesicular body
MS	Mass spectrometry
POPC	Palmitoyl-oleoyl-phosphatidylcholine
proSP-C	Surfactant protein C precursor
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
SP-A	Surfactant protein A
SP-B	Surfactant protein B
SP-C	Surfactant protein C
ThT	Thioflavin T
TM	Transmembrane
TNMD	Tenomodulin

1 Introduction

1.1 BRICHOS domain

The BRICHOS domain was recently discovered by *Sanchez-Pulido et al*, in four different protein families; Bri2, Chondromodulin-1, CA11 and SP-C (Sanchez-Pulido et al., 2002). The definition of a BRICHOS domain is solely based on sequence alignments and since 2002 the number of known proteins with a BRICHOS domain has grown and it has been found in over 300 proteins in 12 distantly related protein families. The proteins have a very wide range of functions and disease associations, spanning from cancer to interstitial lung disease. The twelve different family members in the BRICHOS family are (in the order of sequence similarities to the BRICHOS domain of prosurfactant protein C(proSP-C)): proSP-C, group C, gastrokines 1 and 2 (GKN1, GKN2), group B, chondromodulin (LECT1), tenomodulin (TNMD), a small group of two proteins, group A, integral transmembrane protein 2A, C and B (ITM2A, ITM2C and ITM2B) (Hedlund et al., 2009).

BRICHOS proteins have four different regions; hydrophobic, linker, BRICHOS and C-terminal (fig. 1). The only exception is the proSP-C family, where the BRICHOS domain is the last region. The hydrophobic region shows a high conservation in all families and is thought to be either a transmembrane (TM) region or a signal peptide or it can function as both as in the case of proSP-C (Keller et al., 1991, Conkright et al., 2001). The linker region only shows a high conservation in proSP-C, indicating that this region is of specific importance here. Although the conservation is high within each family, *e.g.* the proSP-C family, there are only three residues that are strictly conserved in all 12 BRICHOS families; one Asp and two

Cys residues and all three are located in the BRICHOS region. The areas around these strictly conserved residues also show a high degree of conservation. The two conserved cysteines form a disulfide bridge in proSP-C (paper IV) and are suggested to form a disulfide bridge in GKN2 (Westley et al., 2005) and this is likely also true for the other families. The C-terminal region is highly conserved in the C-group and is well conserved in ITM2, TNMD and in LECT1. Although the sequence identity between the 12 protein families is low, *e.g.* below 25% sequence identity between human Bri2 and proSP-C, the secondary structure predictions of all 12 families show similar structures. Up until now the structure and function of the BRICHOS domain has been unknown (Hedlund et al., 2009).

Figure 1. **The four different regions in the BRICHOS proteins**; Hydrophobic in green, linker in grey, BRICHOS region in blue and the C-terminal in yellow color.

1.1.1 Pro-surfactant protein C

Surfactant protein C (SP-C) is together with surfactant protein B (SP-B) and phospholipids responsible for lowering the surface tension at the alveolar air-water interface and by doing so preventing the lungs from collapse at end expiration (Whitsett and Weaver, 2002). Human SP-C is encoded by a single gene (*SFTPC)* located on chromosome 8, that contains 6 exons, but only 5 of them are translated (Fisher et al., 1988, Glasser et al., 1988). SP-C is produced in the alveolar type II epithelial cells from a 21 kDa precursor (Beers et al., 1994), proSP-C, which contains four different regions; a short N-terminal segment that is located in the cytosol and is important for intracellular trafficking, a 35 amino acid long TM region that makes up the main part of mature SP-C, a linker region followed by a BRICHOS domain that is located in the endoplasmic reticulum (ER) lumen (fig. 2) (Li et al., 2004, Conkright et al., 2001, Keller et al., 1991).

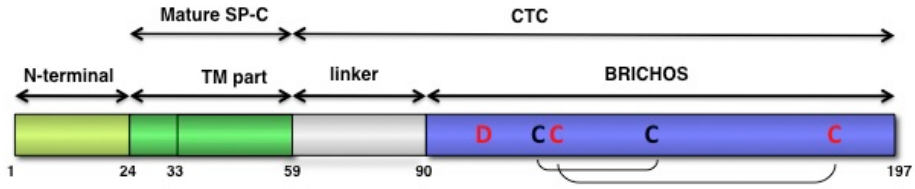

Figure 2. **Schematic structure of proSP-C**. The position of the first residue of each region is indicated. The N-terminal is shown in light green and the transmembrane (TM) and mature SP-C in darker green. The CTC (the C-terminal of proSP-C) is presented in grey for the linker and in blue for the BRICHOS domain. The strictly conserved residues in all BRICHOS families are marked with red color.

The mature SP-C peptide mainly consists of an α-helix. This helix is discordant, *i.e.* it is composed of amino acids with a high β-strand propensity, primarily Ile and Val (Kallberg et al., 2001). Once the helix has formed in the membrane it is stable, but taken out from its phospholipid environment it can unfold and convert into β-sheet aggregates and amyloid-like fibrils (Szyperski et al., 1998, Gustafsson et al., 1999). Synthetic SP-C analogues with the poly-Val stretch, cannot fold into an α-helix conformation, but instead forms insoluble aggregates (Johansson et al., 1995). Therefore leucine (with high helix propensity (Johansson et al., 2010)) was introduced instead, and synthetic SP-C analogues with poly-Leu can fold into a stable helical conformation and these analogues show almost the same surface activity as native SP-C (Palmblad et al., 1999, Nilsson et al., 1998).

Biosynthesis of proSP-C

ProSP-C is synthesized in the alveolar type II cells as an integral membrane protein with a type II orientation in the ER membrane with its C-terminal in the ER lumen and the N-terminal in the cytosol (Keller et al., 1991, Beers et al., 1994). Expression of mature SP-C without its N- and C-terminal parts results in aggregation of mature protein in the secretory pathway, indicating that the proparts are necessary for maturation (Beers et al., 1998, Conkright et al., 2001).

ProSP-C gets posttranslationally modified with palmitate and proteolytically cleaved (fig. 3). The first to happen is palmitoylation of two Cys residues (5 and 6 in the mature peptide, fig. 5). This probably takes

place in the *cis*-Golgi network (Vorbroker et al., 1992). To generate mature SP-C, proteolytic events take place in four steps. The C-terminal domain is removed in two steps, first a 16 kDa intermediate is generated and after that a 7 kDa intermediate. Both cleavages take place in the multi vesicular bodies (MVBs) (Solarin et al., 2001, Beers et al., 1994) as does the third step that removes the first 9 N-terminal residues. The fourth and last step occurs in the lamellar bodies (LBs) where 12 amino acids of the N-terminal get proteolytically cleaved off (Vorbroker et al., 1995, Beers et al., 1994, Johnson et al., 2001, Solarin et al., 2001). Cathepsin H, a cysteine proteinase (Brasch et al., 2002) has been implicated in the processing of proSP-C. This enzyme alone can however not process the proprotein into the mature peptide, which means that there are still unidentified proteases that must be involved in the process. SP-B and SP-C are stored in the LBs where the protein is assembled in densely packed bilayers (Haagsman and van Golde, 1991). The LBs fuse with the plasma membrane and release their contents into the liquid layer that covers the epithelial surface. After this event, the lipid vesicles with proteins gets rearranged into tubular myelin, which is a highly ordered array of phospholipids and proteins, and then inserted into the monolayer (Vorbroker et al., 1995).

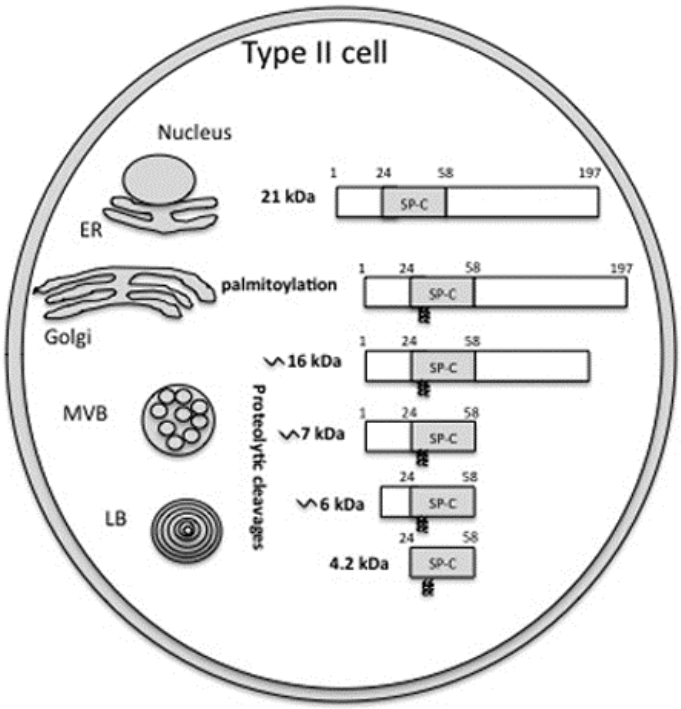

Figure 3. **Biosynthesis in alveolar type II cells**. Human proSP-C is synthesized as a 191-197 amino acid integral TM protein. The precursor protein gets palmitoylated in the Golgi network and proteolytically cleaved in several steps in the MVBs and LBs.

Mutations in the proSP-C gene

Mutations in the *SFTPC* gene are associated with interstitial lung disease (ILD) with intracellular protein aggregates (Nogee et al., 2001, Nogee et al., 2002). There are about 50 known mutations and the majority of them are located in the C-terminal part of the precursor protein (linker and BRICHOS domain) (paper IV). The mutations have been found in patients spanning from newborn to adult and about half of them arise spontaneously and the rest are inherited. The age of onset and the phenotype can differ a lot between individuals that carry the same mutation (Hamvas, 2006).

Some of the *SFTPC* gene mutations have been more thoroughly studied. The most common is the I73T mutation, located in the linker region of proSP-C. Staining lung tissue from patients with proSP-C(I73T) mutation with antibodies against proSP-C reveal a diffuse pattern of the alveolar type II cells, which indicates that this mutation is associated with abnormal trafficking and intracellular accumulation of protein. Immunohistochemical

analyses also showed SP-B, proSP-B, aberrantly processed proSP-C and high levels of SP-A in the lung of affected patients, and the surfactant lipid composition in the alveolar type II cells was also altered. Mutant proSP-C ended up in early endosomes in transfected A549 epithelial cells (Tredano et al., 2004, Brasch et al., 2004, Woischnik et al., 2010). The E66K mutation is also located in the linker region and lung histology studies showed abnormal processing of proSP-C and an increased amount of SP-A. When transfected into A549 cells, proSP-C(E66K) ended up in the early endosomes (Stevens et al., 2005).

Deletion of exon 4 (Δexon4) due to a mutation that affects the splicing of the *SFTPC* gene, results in reduced amounts of proSP-C and a total lack of mature SP-C in the bronchoalveolar lavage fluid (BAL) (Nogee et al., 2001). This is the first proSP-C mutation described. In different cell lines the mutation causes misfolding of the proSP-C, ER stress and induction of the unfolded protein response, leading to disrupted lung morphology (Mulugeta et al., 2007, Bridges et al., 2003). The Δexon4 mutation is located in the BRICHOS region of proSP-C, like the L188Q mutation. This mutation is located next to one of the strictly conserved Cys residues. The age of onset differs a lot between patients in one described kindred, and electron microscopy of affected lungs revealed alveolar type II cells with abnormal lamellar bodies (Thomas et al., 2002). Cells transfected with proSP-C(L188Q) show misfolded proSP-C and upregulation of ER stress markers (Maguire et al., 2010, Lawson et al., 2008).

ProSP-C knock-out mice (strain Swiss black mice) showed mild abnormalities of lung function with decreased stability of surfactant film at low lung volumes (Glasser et al., 2001). Mice of the same strain were treated with bleomycin to induce lung fibrosis and the SP-C knock-out mice showed a greater weight loss, higher mortality and a much slower recovery than the wildtype mice (Lawson et al., 2005). In another mouse strain (Sv strain) the proSP-C knock-out mice get progressive inflammatory lung disease (Glasser et al., 2003). ProSP-C deficiency is thus not lethal and SP-C does not appear to be critical for surfactant function, at least not acutely in mice. The differences observed between mice strains suggest that the phenotype associated with deficiency of proSP-C may be influenced by environmental or genetic factors.

All proSP-C mutations found so far have been on one allele only. Why this leads to lung disease is not clear but possible mechanisms have been suggested. The low levels or total lack of mature SP-C could be the result of a toxic gain of function or a dominant negative effect *i.e* the mutated protein aggregates with the wt protein and this will lead to lung disease (Wang et al., 2003). Alternatively, the production of misfolded proSP-C is difficult to handle for the protein degradation system and this will lead to ER stress, and later on, apoptosis and ILD (Maguire et al., 2010).

1.1.2 Group C

The suggested name for this protein is: BRICHOS domain-containing protein C16orf79 (www.uniprot.org) and the proteins in this group can be found in humans down to *Drosophila melanogaster*. In humans the protein is suggested to be 260 amino acids long and to have a N-glycosylating site. The C-terminal region in this group is highly conserved (Hedlund et al., 2009).

1.1.3 GKN2, GKN1 and group B.

GKN2, GKN1 and group B are closely related and in GKN1 and GKN2 the hydrophobic region may constitute a signal sequence instead of a TM segment (Hedlund et al., 2009).

GKN2

In humans, GKN2 or TFIZ1/GDDR, is a 184 amino acid long pro-protein expressed in gastric mucosa. It contains three Cys residues, two of them are involved in intramolecular binding and one Cys has been shown to form a disulfide bridge with the trefoil factor family 1(TFF1) protein. The TFF1:TFIZ1 heterodimer has been found in gastric mucosa where it could act as a tumor suppressor (Westley et al., 2005). Others have found the protein in mouse and named it blottin. Blottin binds to mouse TFF2 and is found in the gastric epithelial surface and foveolar epithelial cells where it may be involved in gastrointestinal mucosal maintenance (Otto et al., 2006).

GKN1

GKN1 is a 18 kDa pro-protein that is synthesized in cells of the gastric antrum mucosa (Martin et al., 2003). The function of GKN1 is not known but it has a suggested role in mucosal protection and it has also been proven to be down regulated in gastric carcinoma (Oien et al., 2004).

Group B

This group likely represents a novel gastrokine, GKN3 that is synthesized as a 19 kDa proprotein in mice. The gene has become nonfunctional in humans due to a recent mutation. It has been proposed to be associated with gastric atrophy and to take part in the host response to *Helicobacter pylori* in mouse (Menheniott et al., 2010).

1.1.4 LECT1

LECT, Leukocyte cell-derived chemotaxin 1 or chondromodulin, 1 is an abundant protein in fetal bovine cartilage. The protein is synthesized as a 335 amino acid proprotein that gets post-translationally modified by glycosylation and proteolytically cleaved by furin to yield a 28 kDa glycoprotein (Azizan et al., 2001). LECT1 is associated with chondrosarcoma and the expression is reduced in benign cartilage tumors (Hayami et al., 1999) and it also inhibits angiogenesis in cartilage (Hiraki et al., 1997).

1.1.5 TNMD

The 317 residue proprotein is assumed to be a type II membrane protein. It has two glycolsylation sites and it contains eight Cys residues in the C-terminal region. The C-terminal resembles the C-terminal in LECT1 that has been suggested to have an antiangiogenic activity (Shukunami et al., 2001).

1.1.6 Small group of two proteins and group A

None of the proteins in these two groups have been investigated.

1.1.7 ITM2A, ITM2B and ITM2C (Bri proteins)

The Bri proteins can be divided into Bri1 (ITM2A), Bri2 (ITM2B) and Bri3 (ITM2C).

The Bri1 protein

The Bri1 protein is a 263 amino acids long protein that has a possible glycosylation site, like the rest of the proteins in the family. Bri1 has been reported to be involved in chondrogenic differentiation (Deleersnijder et al., 1996, Van den Plas and Merregaert, 2004) Bri1 expression is induced in T cells upon activation and it localizes to the Golgi apparatus, large cytoplasmic vesicles and to the cell surface (Kirchner and Bevan, 1999).

The Bri2 protein

The Bri2 proprotein is a 266 amino acid residue long type II transmembrane protein (fig. 4). The protein gets post-translationally modified by N-glycosylation and proteolytic cleavage at three positions. Furin cleaves in the C-terminal region and this cleavage generates ABri23, a 23 residue peptide (Kim et al., 2002, Kim et al., 1999). The BRICHOS domain is released from the membrane bound N-terminal by ADAM10 and cleavage by SPPL2a/2b releases the intracellular domain (Martin et al., 2008).

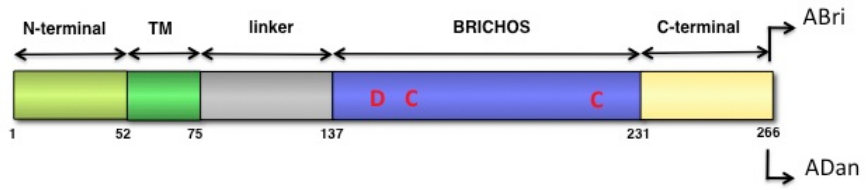

Figure 4. **Schematic structure of Bri2**. The position of the first residue of each region is indicated. The N-terminal is shown in light green and the TM in darker green. The linker region is represented in grey, the BRICHOS domain in blue and the C-terminal region in yellow color. The strictly conserved residues in all BRICHOS families are marked with red color.

The Bri2 protein is associated with familial British and Danish dementia (FBD and FDD). Both diseases have an early onset, median ages of onset, 48 years in FBD and 27 years in FDD. The diseases are similar to Alzheimer's disease (AD) and are characterized by extracellular amyloid plaques with neurodegeneration (Rostagno et al., 2005). A single point mutation gives an Arg codon instead of a stop codon in FBD and this results in a 277 amino acid residue long precursor protein (Vidal et al., 1999). In FDD, an insertion of 10 nucleotides just before the stop codon also results in a 277 amino acid long precursor protein (Vidal et al., 2000). After furin cleavage, a 34 amino acid long amyloidogenic peptide is released in both FBD and FDD (Kim et al., 2002, Kim et al., 1999). The amyloidogenic peptides (ABri in FBD and ADan in FDD) show a 100% sequence identity in their first 22 residues but are completely different in their last 12 residues. Both ABri and ADan isolated from amyloid plaques have been posttranslationally modified at the N-terminus, both peptides have pyroglutamate instead of glutamic acid (Tomidokoro et al., 2005). This modification has been observed in other peptides in brain amyloids *e.g* the Aβ peptide. This modification changes the charge and increases the β-sheet content, which makes both ABri and ADan

more prone to form aggregates and polymerize. Soluble forms of both ABri and ADan have been found in the body fluids of patients and ABri has also been found in systemic amyloid deposits in peripheral tissue (Ghiso et al., 2001).

Lately, ideas to use the Bri2 protein as a possible molecular target for an anti-amyloid therapy for AD have been put forward. Immunohistochemical studies of amyloid deposits in FDD revealed the presence of Aβ, which suggests that these two molecules can interact during misfolding and aggregation, (Tomidokoro et al., 2005, Rostagno et al., 2005). The Bri2 protein has also been shown to interact with the amyloid precursor protein (APP) in cell cultures and the interaction affected the APP processing (Fotinopoulou et al., 2005). The soluble ABri23 peptide has also been shown to bind to monomers and prevent Aβ from forming oligomeric aggregates, both *in vitro* and *in vivo* (Kim et al., 2008, Matsuda et al., 2009b). Finally, a recombinant human BRICHOS domain of Bri2 can bind to $A\beta_{1-40}$ and to the soluble ABri23 peptide *in vitro* (Peng et al., 2010).

The Bri3 protein

The Bri3 is a 267 amino acid long protein of unknown function. It is mostly expressed in the brain as a type II TM protein with an extracellular C-terminal and it gets posttranslationally modified by N-glycosylation (Vidal et al., 2001) and proteolytically cleaved by furin (Wickham et al., 2005). So far, no diseases have been associated with the Bri3 protein. The amino acid sequence of Bri3 is 44% identical with the sequence of Bri2 and since Bri2 has been proven to interact with APP and the Aβ peptide the Bri3 was also studied in a similar way. Bri3 interacts with APP in cell culture and reduces both α- and β-APP cleavage by blocking the access for these secretases (Matsuda et al., 2009a).

1.2 Protein folding and chaperones

1.2.1 Protein folding

Proteins are made up of 20 different naturally occurring amino acid residues. The specific sequence of amino acids is unique for each protein. The residues are connected via peptide bonds and the primary structure of a protein is defined by the order of the residues. The secondary structure, α-helices or β-sheets, is held together by hydrogen bonds between atoms in the backbone of the polypeptide chain. The secondary structure elements

assemble into a three dimensional tertiary structure, stabilized by strong electrostatic and hydrophobic interactions and sometimes by disulfide bonds. The quaternary structure is an assembly of several polypeptide chains.

Protein folding follows thermodynamic principles. Many attempts have been made to establish general folding principles but so far no one has succeeded. It has been shown that the driving force for the folding process is the search for the lowest energy state and in most cases folded proteins represent the most thermodynamically stable conformation, *i.e.* the conformation with the lowest potential energy (Dobson, 2003). During this process the protein forms several different intermediate steps before it finally reaches its native conformation. All different conformations that the protein can adopt during the folding process are associated with free energy. If the free energy is plotted against the corresponding conformation an energy landscape can be created which visualizes the proteins way from an unfolded to a folded state (Plotkin and Onuchic, 2002, Dobson, 2001).

Protein folding in the cell is an even more complicated procedure due to the very crowded environment. The concentration of macromolecules in the cell can be as high as 400 mg/ml and this limits the available volume inside the cell for the growing polypeptide chain (Ellis and Minton, 2003). When the polypeptide chain is synthesized, it exposes hydrophobic regions, which can interact with other hydrophobic cellular constituents and this can lead to a failure for the protein to fold into its functionally native state (Radford, 2000). Misfolding of the protein can result in malfunction and the formation of protein aggregates. Human misfolding diseases originate when the proteins adopt non-native structures, which will lead to aggregation with intra and/or extracellular deposits. Nature has tried to overcome these challenges by a number of systems to support folding and one of these systems are the molecular chaperones (Ellis, 1990).

1.2.2 Molecular Chaperones

All cells need chaperones to assist in the folding of the newly synthesized polypeptide chain, so it does not get involved in inappropriate interactions (Hartl, 1996). Molecular chaperones are currently defined "as a large and diverse group of proteins that share the property of assisting the non-covalent folding and unfolding and the assembly and disassembly of other macromolecular structures, but are not permanent components of these structures when they are performing their normal biological functions" (Ellis, 2006).

Proteins can also misfold or unfold because of cellular stress *e.g.* temperature changes, genetic mutations or ageing. When the cell is under stress the translation of heat shock proteins (HSP) is induced. There are a number of heat shock proteins and almost all of them function as chaperones. The heat shock proteins can be classified into six main families: HSP100, HSP90, HSP70, HSP60, HSP40 and the small HSPs. The classification is based on molecular mass (Muchowski and Wacker, 2005). The HSP70s consist of a C-terminal substrate binding domain (SBD) and an N-terminal ATP-binding domain. The non-native proteins have exposed hydrophobic areas that can bind to the SBD of the HSPs. The binding and release is then repeated in cycles until the protein has folded into its native structure (Beckerman, 2009). HSP70 together with its co-chaperone, HSP40, has been shown to participate in assembly of proteins into macromolecular complexes, aggregation prevention and protein degradation (Broadley and Hartl, 2009). HSP90 together with HSP70 and HSP40, have an early effect in the amyloid β-peptide 1-42 ($A\beta_{1-42}$) fibril formation (Evans et al., 2006).

There are also extracellular chaperones, like apolipoprotein J, also called clusterin. Clusterin has been found to inhibit amorphous aggregation of a number of proteins and to inhibit Aβ and α-synuclein from fibril formation (Humphreys et al., 1999, Yerbury et al., 2007). Haptoglobulin and α_2-macroglobulin are two glycoproteins that also have been suggested to work as extracellular chaperones. Both proteins can bind to $A\beta_{1-42}$, calcitonin (associated with localized amyloidosis), and lysozyme (associated with familial systemic amyloidosis) and inhibit the formation of fibrils *in vitro* (Yerbury et al., 2009). α_2-macroglobulin can also suppress the formation of amorphous aggregates of different proteins under stress (French et al., 2008).

Some chaperones function by minimizing the risk for partially folded proteins to aggregate with each other by offering a platform for the polypeptide to be able to fold without the disturbance from other proteins or cellular elements. These chaperones have little or no sequence specificity for the polypeptide chains that they bind to and are often ATP dependent (Beckerman, 2009). Steric chaperones on the other hand, bind specifically and ad structural (steric) information to the polypeptide chain that is in the process of folding and in this way the chaperones can control the folding. These chaperones are ATP independent. Some of the steric chaperones are localized in the N-terminal part as a prosequence and act as intramolecular

chaperones that bind to the rest of the chain (Inouye, 1991). When the protein has folded correctly the N-terminal chaperone gets cleaved off and degraded. The prosequence chaperones can also be added *in vitro* to partially folded chains in trans and still assist in the folding of the polypeptide chain that have failed to fold and instead adopted a compact, partially folded state that resembles that of a molten globule (Ellis, 1998).

1.3 Misfolding and amyloid

Misfolding of proteins is the cause of almost thirty amyloid diseases in man *e.g.* AD, Creutzfeldt-Jakob disease, Parkinson's disease and type II diabetes (Sipe et al., 2010). Even though the proteins responsible for the diseases do not have any sequence identity they all form amyloid fibrils which have the same morphology. These fibers are insoluble, protease resistant and have β-sheet structure. All amyloid fibers consist of β-strands that runs perpendicular to the axis of the fiber (cross β-structure) (Eanes and Glenner, 1968). The fibers give distinct X-ray signals at 4.7 Å and at 10-11 Å, are unbranched and around 10 nm in diameter (Makin and Serpell, 2005). To be designated amyloid it must occur in tissue deposits, and staining with Congo red should give green birefringence under cross-polarized light (Sipe et al., 2010). There are two different types of the amyloid diseases, localized or systemic. In the localized amyloidosis, only one organ is affected *e.g.* the brain in Alzheimer's disease, while in the systemic amyloidosis, deposits are found in a wide range of tissues and organs *e.g.* lysozyme amyloidosis.

The formation of amyloid fibrils can occur when the native protein is destabilized and has started to form aggregates. Amyloid fibril formation, a nucleation dependent process (Jarrett and Lansbury, 1993), typically has three phases: the lag phase, elongation phase and the equilibrium. The lag phase encompasses the formation of soluble oligomers or nuclei from monomers, which is a rather slow process, and these oligomers are required to seed the fibril growth. This step can be accelerated by the addition of seeds (preformed nuclei). The next step, the elongation phase, is on the other hand rather fast where the oligomers start to form fibrils. In the last steady state phase, the monomers and fibrils are at an equilibrium.

1.3.1 Amyloid β-peptide (Aβ)

AD is a neurodegenerative disorder characterized by cerebral extracellular Aβ deposits and intracellular tangles made of the protein tau. Alois

Alzheimer, a German physician, first described AD in 1907 and the disease is the commonest cause of dementia in elderly (Fratiglioni et al., 2000). The extracellular deposits or senile plaques in the brain of an AD patient, contain amyloid fibrils formed by the Aβ-peptide (Glenner et al., 1984). Aβ is derived from the amyloid-β precursor protein (APP), which is a type I transmembrane protein with the N-terminal located in the extracellular space. APP is processed in several steps in two different pathways; one amyloidogenic and one non-amyloidogenic (Zhang et al., 2011). The amyloidogenic pathway generates Aβ which mainly consists of 40-42 amino acid residues. The first cleavage of APP in the amyloidogenic pathway is performed by β-secretase (Vassar et al., 1999), the second cleavage is performed by γ-sectretase and this generates the Aβ-peptide. The γ-sectretase cleavage is heterogeneous and generates peptides ranging from 38 to 43 residues, the predominant variants are the $A\beta_{1-40}$ and the more aggregation prone $A\beta_{1-42}$ (Younkin, 1995). In the non-amyloidogenic pathway the first step is performed by α-secretase (Sisodia, 1992) and the second step is performed by γ-sectretase (Haass et al., 1993). Mutations in APP (either outside or within the Aβ sequence) or in the presinilins (that are constituents of the γ-sectretase complex) alter the rate and/or the site of APP processing and thereby the generation and aggregation propensity of Aβ peptides and this will cause AD in younger persons as well. Patients with AD first lose their short-term memory followed by disorientation and general recess in cognitive function as the disease progresses. The definitive AD diagnosis can only be made *post mortem* through a pathological exam of the brain where the neuropathological injuries can be established.

The functions of APP and Aβ are still unknown and there is no cure or effective therapy for AD. The therapies available today are only symptomatic, lot of work is being put into this challenging task of finding effective therapies, focusing on *e.g.* decreasing the production of the amyloidogenic peptide by inhibiting the β- or γ-secretases (Ghosh et al., 2008, Fleisher et al., 2008), prevent aggregates from forming by inducing the production of chaperones (Ansar et al., 2007) or using small molecules or peptides as inhibitors of fibril formation (Nakagami et al., 2002). So far none of the strategies has proved to work.

2 Present investigation

2.1 Aims of this thesis

The general aim of this thesis was to characterize and to find a function and binding partners to CTC and its BRICHOS domain.

More specific aims were to:

- ✓ Find a way to express and purify CTC from *E.coli*.
- ✓ Characterize the binding specificity of CTC and its BRICHOS domain towards the proSP-C and mature SP-C *in vitro* and in transfected cells.
- ✓ Generate structural information about the BRICHOS domain.
- ✓ Find out if the BRICHOS domains from proSP-C and Bri2 can act as chaperones against the Aβ-peptide.

2.2 Results

2.2.1 Expression and purification of CTC and proSP-C BRICHOS (paper I and II)

The C-terminal part of human proSP-C, residues 59-197, (CTC) (see figure 5 for sequence) was expressed as a fusion protein with thioredoxin-His_6- and a S-tag in *E.coli*. Since CTC contains two disulfide bridges the Origami strain with an oxidizing environment was used. CTC is mainly found as an insoluble protein even though it is expressed with tags commonly used to increase the solubility. It is probably associated with membranes, rather than present in inclusion bodies since it can be dissolved in 2M of urea, which is less than what is required for CTC denaturation (paper III). After extensive washing on Ni^{2+}-column, removal of the thioredoxin- and His-tag and further purification with ionexchange chromatography, the purity was analysed by SDS-PAGE and nondenaturing PAGE. Also a mutant form of CTC, L188Q that is linked to ILD, was expressed and purified in the same way as the wt protein (paper I). A construct that only contained the postulated BRICHOS domain (residues 94-197) (Sanchez-Pulido et al., 2002) was also attempted to be produced. The expression of proSP-C (94-197) however, gave a low amount of protein which aggregated easily and the construct was therefore later extended to residues 86-197 of proSP-C (Fitzen et al., 2009), and this protein gave a higher yield and did not aggregate (paper II).

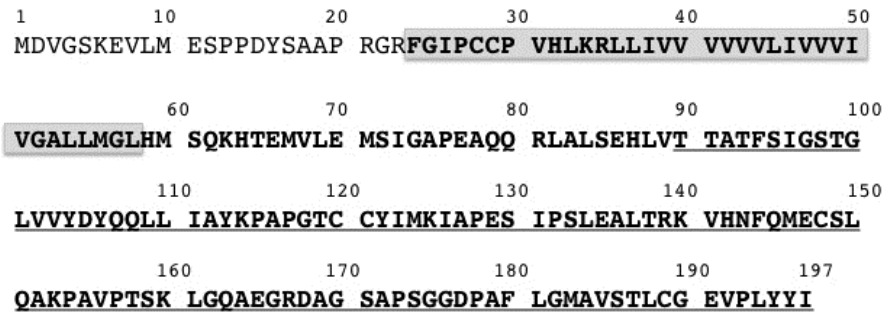

Figure 5. **The human proSP-C sequence**. The first 23 amino acids represent the cytosolic N-terminal part. The amino acids with grey background correspond to the mature SP-C (residues 24-58). Amino acids in bold represents CTC (residues 59-197) and bold and underlined amino acids represent the BRICHOS domain (residues 90-197) as defined from the x-ray structure (paper IV).

2.2.2 Biochemical properties of CTC (paper III)

Recombinant CTC was analyzed for secondary and quaternary structure, domain organization and stability towards denaturation by analytical ultracentrifugation (AUC), mass spectrometry (MS) and circular dichroism (CD) measurements.

AUC showed that CTC is heterogeneous in size with the main species found being a trimer, but monomers, hexamers and nonamers could also be seen, native PAGE also verified this. The quaternary structure was also analyzed with electrospray ionization mass spectrometry (ESI MS) and trimers, hexamers, monomers and even dodecamers were seen.

CTC contains four Cys residues and these have been proposed to form two disulfide bridges. CTC was treated with trypsin and the peptides derived were analyzed with matrix-assisted laser desorption/ionization (MALDI) MS. The fragments obtained showed that one of the two Cys residues at position 120 or 121 forms a disulfide with Cys148 and the other one with Cys189. Since Cys120 and Cys121 are right next to each other it is hard to assign the disulfide pairings any further. However, since Cys121 and Cys189 are strictly conserved across all BRICHOS domains, it is likely that these two can form a disulfide bridge and that the other disulfide bridge is formed between Cys120 and Cys148 (paper III). This was later verified in the x-ray structure of the BRICHOS domain (paper IV).

The stability of the protein was measured in several ways. Trypsin treated CTC was analyzed by MALDI MS and the sensitivity towards trypsin cleavage differed >1000 fold in different parts of the protein. The BRICHOS domain is much more resistant to cleavage than the rest of CTC. CD measurements of reduced and non-reduced CTC showed that the secondary structure is only affected significantly by urea or heat if the disulfides are reduced. Differential scanning calorimetry (DSC) was also used to determine the thermal stability of free and membrane bound CTC. These data indicated that membrane bound CTC is less stable than the free CTC (paper III).

To gain further insight into the structural properties of CTC the interaction with the dye 1,1'-bis(4-anilino-5,5'-naphtahalenesulfonate) (bis-ANS) was measured by fluorescence spectroscopy. The fluorescence intensity of bis-ANS depends on its environment and the intensity was increased when CTC was added, proposing that CTC have hydrophobic

surfaces that bis-ANS can bind to. Since CTC contains six Tyr residues and no Trp, fluorescence resonance energy transfer (FRET) studies were performed to see if these Tyr residues are located close to the bis-ANS binding site. The emission spectrum of CTC after excitation at 280 nm in the presence of increasing amounts of bis-ANS was recorded, and this showed a gradual decrease in Tyr fluorescence with a simultaneous increase in the bis-ANS fluorescence. Since free bis-ANS does not emit when excited at 280 nm this indicates an energy transfer from Tyr to bis-ANS. This result indicates that Tyr residues in CTC are located close to the binding site of bis-ANS (paper III).

These data indicate that CTC is produced in *E.coli* as a folded and stable protein. CTC forms mainly trimers, contains two disulfide bridges and can only be denatured after reduction. CTC has a hydrophobic surface with tyrosine residues located close to a binding site for bis-ANS.

2.2.3 Membrane association, substrate specificity and requirements for peptide binding (paper I, II, IV and V)

Since, in proSP-C, CTC is located near the phospholipid membrane, the ability of CTC to associate with lipids was investigated. CTC can insert into phospholipid monolayers of palmitoyl-oleoyl-phosphatidylcholine (POPC) and dipalmitoyl-phosphatidylcholine (DPPC)/POPC but not into monolayers of DPPC. The mutant L188Q could not insert into any of the monolayers tested. The POPC and DPPC/POPC monolayers are loosely packed, similar to how membranes are packed in the early secretory pathway. DPPC is tightly packed and this is similar to the more ordered membranes in the distal secretory pathway and in particular to lung surfactant (paper IV).

Both CTC and CTC(L188Q) was incubated with lung surfactant phospholipids with and without the SP-C peptide in an α-helical or non-helical conformation. The membrane fraction was collected by centrifugation, and the pellets and supernatants were analyzed by SDS-PAGE and western blot. In the presence of phospholipids only, or phospholipids with α-helical SP-C incorporated, neither wt nor mutant CTC bound to the membrane but in the presence of phospholipids that contained non-helical SP-C, CTC was found in the phospholipid pellet. CTC(L188Q) was still only found in the supernatant, indicating that CTC but not its mutant form can interact with phospholipid membranes with non-helical SP-C incorporated (paper I).

The ability of CTC to change non-helical SP-C into an α-helical conformation was measured by CD. This experiment was done in SDS micelles because of the hydrophobicity of the SP-C peptide. Far UV CD spectra of CTC in SDS micelles, or SDS micelles with α-helical or non-helical SP-C with and without CTC present were recorded. Calculated spectra of CTC plus α-helical or non-helical SP-C were compared to the corresponding spectra that were experimentally determined. The difference between the calculated and experimental spectra of CTC and non-helical SP-C showed a change in conformation that indicated an increase in helical content. The calculated and experimental spectra of CTC and α-helical SP-C did not show any difference. CD measurements with SDS micelles with synthetic SP-C(1-21) (see fig. 5 for sequence) incorporated showed a typical β-strand structure. This peptide consists of the first 21 residues in the mature SP-C peptide. When CTC was added no change in secondary structure was seen (paper I and II).

The substrate specificity of CTC was analyzed in several ways, among others a SPOT membrane was used. A SPOT membrane with overlapping peptide decamers corresponding to the mature SP-C sequence (proSP-C sequence 24-58, see fig. 5) and also some spots with the poly-Val sequence replaced with poly-Ala or poly-Leu was used. A SPOT membrane with the sequence LLIVVVVVVL truncated stepwise by one residue at the time was also analysed to see how many residues that are needed for CTC to bind. CTC binds to the hydrophobic stretches found in the SP-C poly-Val region and it can also bind to poly-Leu, but the replacement of poly-Val with poly-Ala abolishes the binding. CTC binds to peptides that are at least three residues long but the binding is much weaker than to peptides that are five or four residues long. Binding to the SPOT membrane with the proSP-C sequence was also investigated with CTC(L188Q) and with the proSP-C BRICHOS domain, residues 86-197. The BRICHOS domain bound to the same peptides as CTC while the mutant did not bind to any of the peptides (paper II). CTC was also incubated with a SPOT membrane with homodecamers of different amino acids. CTC could bind to stretches of V, I, L, F, M and Y but not to A, W, G, P or T. CTC thus bind mainly to residues that promote membrane insertion according to the biological hydrophobicity scale (Hessa et al., 2005) (paper V). A peptide, KKVVVVVVVKK ($K_2V_7K_2$) was also incubated with CTC and the mixture was analyzed by non-denaturing PAGE where a complex between CTC

and $K_2V_7K_2$ could be seen (paper I). Complexes between $K_2V_7K_2$ and CTC were later confirmed by ESI-MS (Fitzen et al., 2009)(paper IV)

The peptide SP-C33 (SP-C peptide where the valines have been exchanged for leucines) (Nilsson et al., 1998), including the sequence LLLLLLLLILLLILGALL (target peptide), was dissolved in either aqueous formic acid, where the peptide is in non-helical conformation, or in ethanol, where it is α-helical. The peptide was then blotted onto a membrane and incubated with CTC. A non-target peptide (IPCCPV) was used as a control. CTC binds to the target peptide if it is applied in non-helical conformation but not if it is applied in an α-helical conformation (paper V).

Taken together, these experiments show that recombinant CTC and proSP-C BRICHOS(86-197) but not CTC(L188Q) binds to stretches of hydrophobic amino acids that correspond to the TM part of mature SP-C but not to its polar cytosolic part. CTC can also bind to other stretches of residues that promote membrane insertion. The binding of CTC to a non-helical SP-C peptide in micelles results in increased α-helical structure. CTC binds also to peptides in non-helical conformation outside of lipid micelles. Finally, we have shown that CTC, but not a mutant associated with ILD, can associate with fluid phospholipid monolayers similar to those in the early secretory pathway.

2.2.4 ILD is an amyloid disease and chaperone function of CTC and its BRICHOS domain (paper I, II and IV)

The mature SP-C peptide has a discordant helix that has been proven to convert to β-sheet aggregates and amyloid like fibrils *in vitro* (Szyperski et al., 1998, Gustafsson et al., 1999). More than 50 mutations have been found in the proSP-C gene and most of them are located in the C-terminal part (paper IV).

Lung samples from ILD patients with mutant proSP-C were analyzed with congo staining and tissue with the I73T or the Δ91-93 mutation showed green birefringence under polarized light. Immunolabeling of lung tissue sections with an antibody against mature SP-C, but not against N-terminal or C-terminal proparts, stained the congophilic depositions and preabsorption with a synthetic peptide that corresponds to residues 24-41 in mature SP-C abolished the immunoreactivity (paper IV).

CTC can bind to SP-C in non-helical conformation *in vitro* and this interaction resulted in a conformational change (papers I, II and V). Interactions between CTC and target proteins were also investigated in cell cultures. Stably transfected HEK293 cells expressing either wt proSP-C or the L188Q mutant, were transiently transfected with CTC carrying a signal peptide from proSP-B, directing it to the ER lumen. The mutant protein is apparently degraded rapidly and only a small fraction can be detected. When transfected with CTC a significant increase of proSP-C(L188Q) could be detected by western blot. The transfected cells expressing wt proSP-C did not show any difference in the amount of protein detected. This result indicates that CTC can bind to mutant proSP-C *in trans* and thereby stabilize it and decrease the degradation (paper I).

Further on, HEK293 cells, stably transfected with proSP-C(1-58) (see fig. 5 for sequence) and CTC with a signal peptide, or cells expressing only proSP-C(1-58) were analyzed. In cells that only express proSP-C(1-58), no protein could be detected in the cell lysate nor in the SDS insoluble/formic acid soluble fraction. Treating the cells with a proteasome inhibitor resulted in small amounts of detectable proSP-C(1-58) in the SDS-soluble fraction, indicating that the protein is synthesized but degraded rapidly. However in cells expressing both proSP-C(1-58) and CTC with signal peptide, proSP-C (1-58) is stabilized and rescued from degradation (paper II).

In conclusion, we have shown that ILD due to mutations in proSP-C is a new amyloid disease and this is likely due to malfunction of the BRICHOS chaperone domain. Moreover, CTC can stabilize mature SP-C in HEK293 cells *in trans* and by doing so hinder it from degradation.

2.2.5 Structure of proSP-C BRICHOS and model of Bri2 BRICHOS (paper IV and VI)

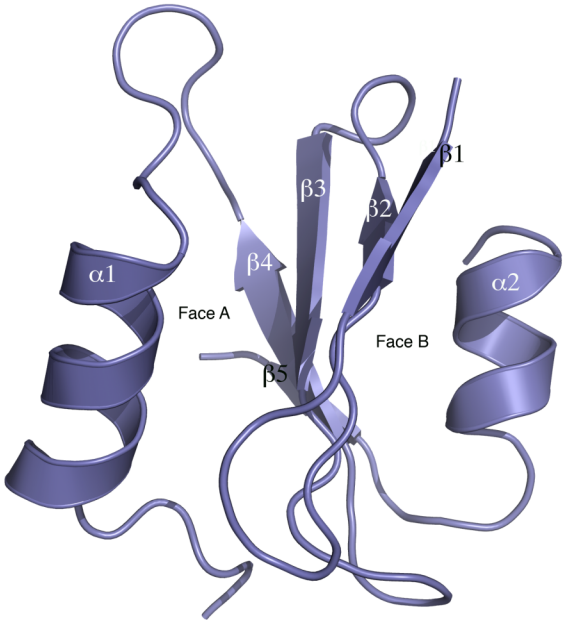

Figure 6. **The crystal structure of the proSP-C BRICHOS monomer**. The structure shows a novel fold with a central five stranded β-sheet with one α-helix on each side.

The structure of trypsin treated CTC was solved to 2.1 Å. There are no structural homologues in the protein data bank and the fold of the BRICHOS domain from proSP-C has not been observed before. The monomer structure shows a central β-sheet made up of five strands, β1-β4 are antiparallel and β4 and β5 are parallel, with one α-helix on either side, one on face A and the other on face B (fig. 6). The two helices are amphiphilic, with the hydrophobic side packed against the β-sheet (α1) and the other side either exposed to the solvent or buried in the trimer interface (α2). The proSP-C residues 149-180 are situated in a presumably flexible loop that showed little electron density and were therefore not modeled in the structure. This loop region was also cleaved by trypsin present during crystallization.

The residues conserved between proSP-C from different species coincide with many of the ILD mutations in CTC. The D105 is a strictly conserved residue in all BRICHOS domains, and mutation of it is associated with ILD. This residue is located in a partially hydrophobic environment and is

in contact with the N-terminal of helix 2. Molecular dynamics (MD) simulations were carried out on the wt monomer and trimer and on a mutant monomer and trimer where the Asp had been replaced with an Asn. In the mutant monomer no major conformational changes occur, however in the wt monomer α2 unwinds and this eventually results in a movement of α1 out from face A, leaving the hydrophobic core accessible to the solvent or possibly for substrate binding (fig. 7). The MD simulations of wt and mutant trimers did not show any unfolding, which indicates that the trimer state of the BRICHOS domain does not expose any hydrophobic surfaces and is unable to bind hydrophobic substrates.

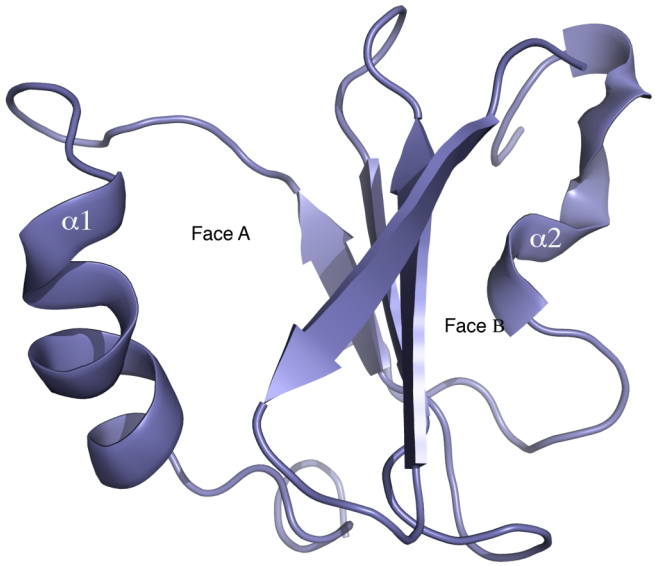

Figure 7. **The structure of proSP-C BRICHOS after MD simulations.** Helix 2 has unfolded and this has lead to the movement of α1 out from face A.

The secondary structure predictions of all BRICHOS domains are very similar, and a homology model of the Bri2 BRICHOS domain could be built with the X-ray structure from the proSP-C BRICHOS as a template. In the central β-sheet there are some highly conserved residues located on face A but there are also some interesting differences from the proSP-C BRICHOS domain. In particular some hydrophobic residues in proSP-C BRICHOS have been replaced with charged residue in Bri2.

2.2.6 Chaperone function of BRICHOS domains against Aβ (paper V and VI)

Molecular chaperones have been proven to have effects on, for example, the Aβ fibril formation and Nerelius et al. showed that CTC could bind to and

stop the fibrillation of both Aβ and medin (Nerelius et al., 2009), and the interaction with Aβ was further characterized.

Synthetic $A\beta_{1-40}$ was incubated with or without CTC for seven days at 37°C with agitation. Aliquots were taken out at the beginning and after seven days, centrifuged and the supernatants were analyzed by SDS PAGE and with electron microscopy. The gels show both Aβ and CTC at the beginning, but after seven days Aβ without CTC has aggregated. If incubated with CTC, the supernatant still contains Aβ after seven days. Electron microscopy showed that Aβ alone has formed fibrils after seven days but in the sample incubated with CTC no fibrils could be seen (paper V).

For further experiments, recombinant peptides that starts with a methionine followed by human Aβ were used (Walsh et al., 2009). The Bri2 BRICHOS protein used corresponds to recombinant human Bri2, residues 90-236, expressed and purified from *E.coli* (Peng et al., 2010). The fibrillation of Aβ(M1-40) and Aβ(M1-42), incubated with and without CTC or Bri2 BRICHOS at different concentrations was followed by thioflavin T (ThT) fluorescence. The lag time for Aβ(M1-40) was highly affected by both CTC and Bri2 BRICHOS and the effects are seen far below equimolar concentrations for both CTC and Bri2 BRICHOS. The effect on Aβ(M1-42) is not as pronounced as for Aβ(M1-40) and higher amounts of both CTC and Bri2 BRICHOS are needed. These experiments show that both proteins are potent inhibitors of Aβ fibril formation. A stopping experiment was also performed where CTC or Bri2 BRICHOS was added at different time points to the fibrillating Aβ peptide. If the BRICHOS proteins were added during the lag time the aggregation was delayed. If the proteins were added during the early part of the elongation phase, the aggregation halted, but if BRICHOS was added late in the elongation phase nothing happened.

The change in secondary structure during Aβ fibril formation, was followed by CD. CD spectra of Aβ(M1-40) alone show a gradual transition from random coil to β-sheet structure, but if any of the BRICHOS proteins were added, the transition is significantly delayed.

SEC was performed on Aβ(M1-40) alone or incubated with CTC or Bri2 BRICHOS at 37 °C for 20 hours. After 20 hour Aβ alone has formed fibrils but Aβ incubated with BRICHOS proteins still mainly migrates as a

monomer with a small fraction eluting together with CTC or with Bri2 BRICHOS (paper VI).

2.2.7 A possible mechanism for the chaperone activity of proSP-C BRICHOS (paper VI)

The linker region in proSP-C is highly conserved but this region is missing in the crystal structure. Hydrogen deuterium exchange mass spectrometry (HDX-MS) was performed on the peptides: $K_2V_7K_2$, KKVVVVVKK ($K_2V_5K_2$) and KKAAAAAAAKK ($K_2A_7K_2$), incubated with CTC or proSP-C BRICHOS. $K_2V_7K_2$ and $K_2V_5K_2$ but not $K_2A_7K_2$ became protected against HDX exchange in the presence of CTC. When the poly-Val peptides bound to CTC the linker region also got protected from HDX (paper IV). These results indicate that the linker region is protected from HDX when the peptides are bound to CTC. A possible mechanism during proSP-C biosynthesis is that the linker region interacts with the TM region and forms a strand-loop-strand structure (β-hairpin) bound to the BRICHOS domain. Once the TM segment has folded into an α-helix the BRICHOS domain is released from the membranes and can be cleaved off by proteolysis (fig. 8).

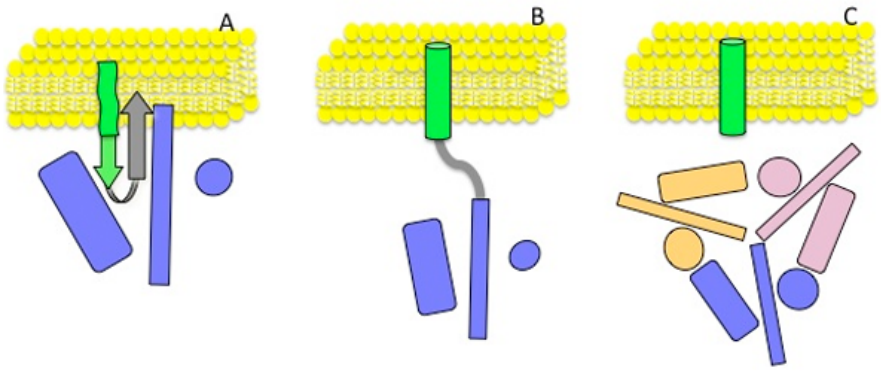

Figure 8. **Postulated mechanism of proSP-C BRICHOS.** The linker region and parts of the TM region form a β-hairpin structure that binds to face A in the BRICHOS domain (A). Once the TM region has formed an α-helix, the BRICHOS domain is released (B). The C-terminal region of proSP-C gets proteolytically cleaved off and the BRICHOS domain can form a homotrimer (C).

2.3 Discussion

Hydrophobicity and low overall charge are two determinants for amyloid formation (Chiti et al., 2002) and are also hallmarks of TM regions (paper V). It has been suggested that VIFC biased segments (segments rich in Val, Ile, Phe and Cys) will promote the formation of inter- or intra-molecular β-sheets and that LAM biased segments (rich in Leu, Ala and Met) will promote the formation of α-helices. Isolated VIFC regions were found in amyloid cores in many of the proteins associated with diseases in man (Johansson et al., 2010). Leu and Ala are common in TM segments (Persson and Argos, 1994) and this is suggested to reduce the risk of forming β-sheets during biosynthesis (Johansson et al., 2010). The α-helix in mature SP-C is however essentially composed of a stretch of 13 Val or Ile (fig. 5)(Johansson et al., 1994). Val and Ile are over-represented in β-sheet and under-represented in α-helix structure, which may make α-helix formation in SP-C less efficient (Mingarro et al., 2000). When folded and inserted into the membrane the α-helix is stable, but in solutions it is unstable and once it has unfolded it will spontaneously convert to β-sheet aggregates and amyloid-like fibrils (Szyperski et al., 1998, Gustafsson et al., 1999), and we show in paper IV that the SP-C peptide can form amyloid in the lung of ILD patients. Nature's way of solving the problem of forming a discordant helix and keeping it from aggregation seems to be a BRICHOS chaperone domain in the C-terminal region of proSP-C, described in this thesis.

CTC, CTC(L188Q), proSP-C BRICHOS(86-197) and Bri2 BRICHOS (90-236) have been produced in *E.coli*. Both CTC and CTC(L188Q) needed to be dissolved in 2M of urea probably because of their association with membranes. Both CTC and CTC(L188Q) could thereafter be purified in a soluble state. ProSP-C BRICHOS(94-197) was purified to a low yield and this was probably because of the lack of the first four amino acids in the BICHOS domain. In support of this, when extended to proSP-C BRICHOS (86-197) the protein yield increased. Bri2 BRICHOS (90-236) does not need to be purified with 2M of urea, probably because it is not associated with membranes.

Since mature SP-C is one of the most hydrophobic peptides known a lipid environment was needed for its analysis and surfactant lipids, SDS micelles and mixtures of DPPC and POPC have been used. The binding of CTC to non-helical SP-C in a lipid environment resulted in a conformational change. The binding of CTC to non-helical SP-C could be due to the fact that non-helical SP-C does not insert into the lipid

environment and is therefore accessible for CTC to bind. However it was later shown that CTC binds to target peptides in non-helical conformation attached to membranes but not to the same peptide if it is in α-helical conformation. The change in secondary structure seen with CTC and non-helical SP-C could occur in CTC and this possibility was investigated further. A peptide (SP-C1-21) that lacks the second half of the SP-C poly-Val stretch, does not give any change in secondary structure. This peptide contains a stretch of hydrophobic residues to which CTC can bind, but is not long enough to also allow a stretch of nonbound Val in which a conformational change could take place. This indicates that the change in secondary structure seen occurs in the peptide and not in CTC.

In the lung, the natural target substrate for CTC is likely the proSP-C TM region and we have shown that CTC can bind to SP-C and to poly-Val sequences in lipids, in solution and bound to SPOT membranes. But the CTC substrate specificity stretches beyond a poly-Val sequence, and this broad substrate specificity could be explained by its function. CTC must be able to bind to any segment within the proSP-C TM region that is not in α-helical conformation to prevent β-sheet aggregation.

The crystal structure of proSP-C was solved after years of attempts to obtain useful crystals. Residues 149-180 in proSP-C is probably in a flexible loop and not until this loop was cleaved off with trypsin did the BRICHOS domain form crystals. Trypsin treated CTC can associate with monlayers of POPC and POPC/DPPC as the wildtype, show the same CD spectrum as CTC and also binds to a triple Val peptide. These result show that treatment with trypsin has not affected the structure significantly and that the flexible loop is not required for the substrate binding. Many of the conserved residues are located on face A and B and these could be of importance in the substrate binding. Interestingly many of the conserved residues in Bri2 are likewise located on face A and B, but on face A some of the hydrophobic residues are exchanged for residues with more polar side chains. These exchanges correlate well with the target substrates of proSP-C and Bri2 BRICHOS, proSP-C BRICHOS binds to highly hydrophobic peptides (papers I, II, IV and V) while Bri2 BRICHOS binds to more polar peptides (Peng et al., 2010).

Asp 105 appears to be of importance by destabilizing α2 in the monomer and this leads to a move in α1 away from face A, leaving it open for substrates to bind (paper IV). FRET experiments showed that energy could

be transformed from Tyr residues in CTC to bound bis-ANS (paper III) and there are four Tyr residues situated on face A. These data suggest that face A is the substrate binding site, but this needs to be verified by determination of a structure of a substrate-BRICHOS complex.

In the asymmetric unit of the crystals, there are two trimers and a trimer state has been observed before with AUC and native PAGE. This seems to be a stable state and the MD simulations of the trimer showed that α-helix 2 did not unfold and no move of α-helix 1 was observed. It is hard to imagine BRICHOS to act as a chaperone as a trimer, and a small monomer fraction has been seen with native PAGE, MS and AUC. It was also shown that oligo-Val peptides bind to monomeric proSP-C BRICHOS (Fitzen et al., 2009). Using DSC, it was seen that the membrane bound CTC is less stable than CTC in solution and this could indicate that the membrane bound CTC is a monomer, but this needs to be experimentally verified. Others have shown by chemical cross linking of proSP-C in A549 cells that CTC does not oligomerize (Wang et al., 2002). We suggest that the BRICHOS domain exerts its function in a monomer state but the trimer state is important as a "capped" state. The proSP-C BRICHOS domain can bind to hydrophobic stretches and in the crowded interior of the cell capping is needed to avoid inadvertent binding to any hydrophobic regions. A capping mechanism is utilized by many chaperones, like the sHSPs (Haslbeck et al., 2005).

The proSP-C TM region has a high barrier for α-helix formation since it mainly consists of Val residues (Hosia et al., 2002, Szyperski et al., 1998). When BRICHOS binds to the TM region the energy barrier is suggested to get reduced, making formation of an α-helix energetically favorable (paper II). BRICHOS thus both assists the TM region to fold correctly and prevents it from forming aggregates. This is compatible with BRICHOS being an intramolecular, steric chaperone. Membrane-integrated chaperones that bind to TM regions have been described. These chaperones bind to TM helices and prevent them from aggregating by holding them until the TM helices find their right binding partner (Kota and Ljungdahl, 2005). ProSP-C BRICHOS differs from these chaperones by not being an integral membrane protein and by binding to nonhelical conformations.

It has been shown (Peng et al., 2010, Nerelius et al., 2009) that both recombinant CTC and Bri2 BRICHOS bind to Aβ. We have confirmed that BRICHOS domains of proSP-C and Bri2 bind to Aβ, which then

aggregates significantly slower than it does on its own. The main effect is seen on the lag phase and we suggest that BRICHOS binds to dimeric and oligomeric Aβ species on pathway to fibril formation. The interaction between BRICHOS and Aβ increases the rate of oligomer dissociation and this result in a sustained pool of monomeric Aβ. Others have described similar mechanisms for HSP 70 that is active in sub stoichiometric amounts and interact with intermediates formed in the fibril formation process (Evans et al., 2006). Keeping Aβ in a monomeric form seems attractive from a therapeutic point-of-view, independently of whether fibrils or oligomers are considered to be the toxic species (Hardy, 2002, Hardy and Higgins, 1992).

We have shown that the BRICHOS domain from Bri2 seems to be more effective towards Aβ than the BRICHOS domain from proSP-C. This is probably due to the difference in sequence specificity between proSP-C BRICHOS and Bri2 BRICHOS. Bri2 BRICHOS has more charged residues than proSP-C BRICHOS in the postulated binding site and binds better to charged peptides. Peng *et al.* showed that recombinant Bri2 BRICHOS binds to the ABri23 peptide (Peng et al., 2010). This peptide shows a strand-loop-strand conformation in secondary structure predictions and a strand-loop-strand conformation is needed in Aβ for the formation of cytotoxic oligomers and fibrils (Sandberg et al., 2010). Thus, this seems to be a common binding motif for the proteins in the BRICHOS family since all proteins (except group A and proSP-C) in this family has a C-terminal region with a suggested strand-loop-strand conformation (Hedlund et al., 2009). The TFF1 contains a strand-loop-strand motif (Polshakov et al., 1997) and this motif may be recognized by GKN2, which has been shown to bind to TFFI (Westley et al., 2005).

There are over 50 known mutations in the proSP-C gene, most of them are located in the BRICHOS domain and many of them co-localize with conserved residues. Amyloid fibrils have been seen in the lung samples from patients suffering from ILD. In these cases the proSP-C gene (*SFTPC*) carried a mutation, either the I73T mutation or the Δ91-93 deletion mutation. The I73T mutation is located in the linker region and this mutation could maybe abolish the binding of the postulated β-hairpin motif to the BRICHOS domain, or prevent it from forming. The Δ91-93 deletion mutation is located in the BRICHOS domain in the trimer interface. This could hinder the trimer from capping the hydrophobic monomer surface, which then binds inappropriately to hydrophobic

constituents. The capping mechanism proposed for proSP-C BRICHOS resembles that of the sHSPs, which exist in an equilibrium between oligomeric and dimeric states. The packaging of sHSPs into dimeric units has been suggested as a control mechanism and the exposure of hydrophobic surfaces in the monomeric state may be responsible for chaperone activity (Benesch et al., 2008). Mutations in the sHSPs has been shown to increase the chaperone activity and enhanced binding to substrate proteins, and these mutations also resulted in an increase of the monomer state which might result in unwanted formation of sHSP-substrate complexes, possibly interfering with their normal function (Almeida-Souza et al., 2010).

3 Conclusions and future perspectives

3.1 Conclusions

- ✓ CTC can be produced in *E.coli* and purified into a folded protein. CTC forms mainly trimers, contains two disulfide bridges and can only be denatured after reduction.

- ✓ CTC and its BRICHOS domain bind to stretches of hydrophobic amino acids that correspond to the TM part of mature SP-C. CTC can also bind to other stretches of residues that promote membrane insertion and CTC can stabilize mature SP-C in HEK293 cells *in trans* and by doing so hinder it from degradation.

- ✓ ILD caused by mutations in the proSP-C gene is a novel amyloid disease.

- ✓ The proSP-C BRICHOS structure shows a central β-sheet made up of five strands with one α-helix on either side. The two helices are amphiphilic, with the hydrophobic side packed against the β-sheet and the other side either exposed to the solvent or buried in the trimer interface.

- ✓ The BRICHOS domains from proSP-C and Bri2 can bind to Aβ and keep it from forming fibrils for a long period of time.

3.2 Future perspectives

Aggregation and misfolding of proteins is linked to a large number of diseases either by a loss of function or gain of toxic function. Nature has tried to solve the problem with disease causing aggregating proteins by a number of strategies, one of them being the molecular chaperones. This thesis has described a novel anti-amyloid chaperone, the BRICHOS domain. Since this BRICHOS domain has been shown to inhibit the Aβ peptide from forming fibrils and to inhibit medin from forming fibrils (Nerelius et al., 2009), this domain might be interesting from a therapeutic point-of-view, a possibility that deserves further studies.

It is suggested that the BRICHOS monomer state is the active state and finding proof of this possibility, *e.g.* by mutating the D105 or chemically cross-linking the monomers into trimers and investigate activativity would be interesting. Proximity ligation assay (PLA) in cell lines to investigate if the trimer or monomer exists *in vivo* likewise seems warranted.

Most of the analysis so far performed, have been *in vitro* experiments. To test the BRICHOS domain in animal models is required for further understanding of its function. The effect of the BRICHOS domain in a *Drosophila* model that express Aβ has been initiated and treating transgenic Aβ-expressing mice with BRICHOS domain is tempting.

The substrate binding surface of the proSP-C BRICHOS domain has only been deduced from structure-function analyses, and its exact nature need to be verified, *e.g.* by solving a structure with bound substrate with X-ray diffraction or NMR methods. There are lot of mutations in the proSP-C gene and how these affect the structure is still to a large extent unknown.

The structural data of Bri2 BRICHOS are based on the model from the proSP-C BRICHOS structure, and the structure of Bri2 BRICHOS domain, like those of all other BRICHOS domains, remain to be experimentally studied.

4 References

ALMEIDA-SOUZA, L., GOETHALS, S., DE WINTER, V., DIERICK, I., GALLARDO, R., VAN DURME, J., IROBI, J., GETTEMANS, J., ROUSSEAU, F., SCHYMKOWITZ, J., TIMMERMAN, V. & JANSSENS, S. 2010. Increased monomerization of mutant HSPB1 leads to protein hyperactivity in Charcot-Marie-Tooth neuropathy. *J Biol Chem,* 285**,** 12778-86.

ANSAR, S., BURLISON, J. A., HADDEN, M. K., YU, X. M., DESINO, K. E., BEAN, J., NECKERS, L., AUDUS, K. L., MICHAELIS, M. L. & BLAGG, B. S. 2007. A non-toxic Hsp90 inhibitor protects neurons from Abeta-induced toxicity. *Bioorg Med Chem Lett,* 17**,** 1984-90.

AZIZAN, A., HOLADAY, N. & NEAME, P. J. 2001. Post-translational processing of bovine chondromodulin-I. *J Biol Chem,* 276**,** 23632-8.

BECKERMAN, M. 2009. Chaperones, Endoplasmic Reticulum Stress, and the Unfolded Protein Response. *Cellular signaling in Health and Disease.* Springer Science + Business Media.

BEERS, M. F., KIM, C. Y., DODIA, C. & FISHER, A. B. 1994. Localization, synthesis, and processing of surfactant protein SP-C in rat lung analyzed by epitope-specific antipeptide antibodies. *J Biol Chem,* 269**,** 20318-28.

BEERS, M. F., LOMAX, C. A. & RUSSO, S. J. 1998. Synthetic processing of surfactant protein C by alevolar epithelial cells. The COOH terminus of proSP-C is required for post-translational targeting and proteolysis. *J Biol Chem,* 273**,** 15287-93.

BENESCH, J. L., AYOUB, M., ROBINSON, C. V. & AQUILINA, J. A. 2008. Small heat shock protein activity is regulated by variable oligomeric substructure. *J Biol Chem,* 283**,** 28513-7.

BRASCH, F., GRIESE, M., TREDANO, M., JOHNEN, G., OCHS, M., RIEGER, C., MULUGETA, S., MULLER, K. M., BAHUAU,

M. & BEERS, M. F. 2004. Interstitial lung disease in a baby with a de novo mutation in the SFTPC gene. *Eur Respir J,* 24**,** 30-9.

BRASCH, F., TEN BRINKE, A., JOHNEN, G., OCHS, M., KAPP, N., MULLER, K. M., BEERS, M. F., FEHRENBACH, H., RICHTER, J., BATENBURG, J. J. & BUHLING, F. 2002. Involvement of cathepsin H in the processing of the hydrophobic surfactant-associated protein C in type II pneumocytes. *Am J Respir Cell Mol Biol,* 26**,** 659-70.

BRIDGES, J. P., WERT, S. E., NOGEE, L. M. & WEAVER, T. E. 2003. Expression of a human surfactant protein C mutation associated with interstitial lung disease disrupts lung development in transgenic mice. *J Biol Chem,* 278**,** 52739-46.

BROADLEY, S. A. & HARTL, F. U. 2009. The role of molecular chaperones in human misfolding diseases. *FEBS Lett.*

CHITI, F., CALAMAI, M., TADDEI, N., STEFANI, M., RAMPONI, G. & DOBSON, C. M. 2002. Studies of the aggregation of mutant proteins in vitro provide insights into the genetics of amyloid diseases. *Proc Natl Acad Sci U S A,* 99 Suppl 4**,** 16419-26.

CONKRIGHT, J. J., BRIDGES, J. P., NA, C. L., VOORHOUT, W. F., TRAPNELL, B., GLASSER, S. W. & WEAVER, T. E. 2001. Secretion of surfactant protein C, an integral membrane protein, requires the N-terminal propeptide. *J Biol Chem,* 276**,** 14658-64.

DELEERSNIJDER, W., HONG, G., CORTVRINDT, R., POIRIER, C., TYLZANOWSKI, P., PITTOIS, K., VAN MARCK, E. & MERREGAERT, J. 1996. Isolation of markers for chondro-osteogenic differentiation using cDNA library subtraction. Molecular cloning and characterization of a gene belonging to a novel multigene family of integral membrane proteins. *J Biol Chem,* 271**,** 19475-82.

DOBSON, C. M. 2001. Protein folding and its links with human disease. *Biochem Soc Symp***,** 1-26.

DOBSON, C. M. 2003. Protein folding and misfolding. *Nature,* 426**,** 884-90.

EANES, E. D. & GLENNER, G. G. 1968. X-ray diffraction studies on amyloid filaments. *J Histochem Cytochem,* 16**,** 673-7.

ELLIS, R. J. 1990. The molecular chaperone concept. *Semin Cell Biol,* 1**,** 1-9.

ELLIS, R. J. 1998. Steric chaperones. *Trends Biochem Sci,* 23**,** 43-5.

ELLIS, R. J. 2006. Molecular chaperones: assisting assembly in addition to folding. *Trends Biochem Sci,* 31**,** 395-401.

ELLIS, R. J. & MINTON, A. P. 2003. Cell biology: join the crowd. *Nature,* 425**,** 27-8.

EVANS, C. G., WISEN, S. & GESTWICKI, J. E. 2006. Heat shock proteins 70 and 90 inhibit early stages of amyloid beta-(1-42) aggregation in vitro. *J Biol Chem,* 281**,** 33182-91.

FISHER, J. H., EMRIE, P. A., DRABKIN, H. A., KUSHNIK, T., GERBER, M., HOFMANN, T. & JONES, C. 1988. The gene encoding the hydrophobic surfactant protein SP-C is located on 8p and identifies an EcoRI RFLP. *Am J Hum Genet,* 43**,** 436-41.

FITZEN, M., ALVELIUS, G., NORDLING, K., JORNVALL, H., BERGMAN, T. & JOHANSSON, J. 2009. Peptide-binding specificity of the prosurfactant protein C Brichos domain analyzed by electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom,* 23**,** 3591-8.

FLEISHER, A. S., RAMAN, R., SIEMERS, E. R., BECERRA, L., CLARK, C. M., DEAN, R. A., FARLOW, M. R., GALVIN, J. E., PESKIND, E. R., QUINN, J. F., SHERZAI, A., SOWELL, B. B., AISEN, P. S. & THAL, L. J. 2008. Phase 2 safety trial targeting amyloid beta production with a gamma-secretase inhibitor in Alzheimer disease. *Arch Neurol,* 65**,** 1031-8.

FOTINOPOULOU, A., TSACHAKI, M., VLAVAKI, M., POULOPOULOS, A., ROSTAGNO, A., FRANGIONE, B., GHISO, J. & EFTHIMIOPOULOS, S. 2005. BRI2 interacts with amyloid precursor protein (APP) and regulates amyloid beta (Abeta) production. *J Biol Chem,* 280**,** 30768-72.

FRATIGLIONI, L., LAUNER, L. J., ANDERSEN, K., BRETELER, M. M., COPELAND, J. R., DARTIGUES, J. F., LOBO, A., MARTINEZ-LAGE, J., SOININEN, H. & HOFMAN, A. 2000. Incidence of dementia and major subtypes in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. *Neurology,* 54**,** S10-5.

FRENCH, K., YERBURY, J. J. & WILSON, M. R. 2008. Protease activation of alpha2-macroglobulin modulates a chaperone-like action with broad specificity. *Biochemistry,* 47**,** 1176-85.

GHISO, J. A., HOLTON, J., MIRAVALLE, L., CALERO, M., LASHLEY, T., VIDAL, R., HOULDEN, H., WOOD, N., NEUBERT, T. A., ROSTAGNO, A., PLANT, G., REVESZ, T. & FRANGIONE, B. 2001. Systemic amyloid deposits in familial British dementia. *J Biol Chem,* 276**,** 43909-14.

GHOSH, A. K., KUMARAGURUBARAN, N., HONG, L., KULKARNI, S., XU, X., MILLER, H. B., REDDY, D. S., WEERASENA, V., TURNER, R., CHANG, W., KOELSCH, G. & TANG, J. 2008. Potent memapsin 2 (beta-secretase) inhibitors: design, synthesis, protein-ligand X-ray structure, and in vivo evaluation. *Bioorg Med Chem Lett,* 18**,** 1031-6.

GLASSER, S. W., BURHANS, M. S., KORFHAGEN, T. R., NA, C. L., SLY, P. D., ROSS, G. F., IKEGAMI, M. & WHITSETT, J. A. 2001. Altered stability of pulmonary surfactant in SP-C-deficient mice. *Proc Natl Acad Sci U S A,* 98**,** 6366-71.

GLASSER, S. W., DETMER, E. A., IKEGAMI, M., NA, C. L., STAHLMAN, M. T. & WHITSETT, J. A. 2003. Pneumonitis and emphysema in sp-C gene targeted mice. *J Biol Chem,* 278**,** 14291-8.

GLASSER, S. W., KORFHAGEN, T. R., PERME, C. M., PILOT-MATIAS, T. J., KISTER, S. E. & WHITSETT, J. A. 1988. Two SP-C genes encoding human pulmonary surfactant proteolipid. *J Biol Chem,* 263**,** 10326-31.

GLENNER, G. G., WONG, C. W., QUARANTA, V. & EANES, E. D. 1984. The amyloid deposits in Alzheimer's disease: their nature and pathogenesis. *Appl Pathol,* 2**,** 357-69.

GUSTAFSSON, M., THYBERG, J., NASLUND, J., ELIASSON, E. & JOHANSSON, J. 1999. Amyloid fibril formation by pulmonary surfactant protein C. *FEBS Lett,* 464**,** 138-42.

HAAGSMAN, H. P. & VAN GOLDE, L. M. 1991. Synthesis and assembly of lung surfactant. *Annu Rev Physiol,* 53**,** 441-64.

HAASS, C., HUNG, A. Y., SCHLOSSMACHER, M. G., TEPLOW, D. B. & SELKOE, D. J. 1993. beta-Amyloid peptide and a 3-kDa fragment are derived by distinct cellular mechanisms. *J Biol Chem,* 268**,** 3021-4.

HAMVAS, A. 2006. Inherited surfactant protein-B deficiency and surfactant protein-C associated disease: clinical features and evaluation. *Semin Perinatol,* 30**,** 316-26.

HARDY, J. 2002. Testing times for the "amyloid cascade hypothesis". *Neurobiol Aging,* 23**,** 1073-4.

HARDY, J. A. & HIGGINS, G. A. 1992. Alzheimer's disease: the amyloid cascade hypothesis. *Science,* 256**,** 184-5.

HARTL, F. U. 1996. Molecular chaperones in cellular protein folding. *Nature,* 381**,** 571-9.

HASLBECK, M., FRANZMANN, T., WEINFURTNER, D. & BUCHNER, J. 2005. Some like it hot: the structure and function of small heat-shock proteins. *Nat Struct Mol Biol,* 12**,** 842-6.

HAYAMI, T., SHUKUNAMI, C., MITSUI, K., ENDO, N., TOKUNAGA, K., KONDO, J., TAKAHASHI, H. E. & HIRAKI, Y. 1999. Specific loss of chondromodulin-I gene expression in chondrosarcoma and the suppression of tumor angiogenesis and growth by its recombinant protein in vivo. *FEBS Lett,* 458**,** 436-40.

HEDLUND, J., JOHANSSON, J. & PERSSON, B. 2009. BRICHOS - a superfamily of multidomain proteins with diverse functions. *BMC Res Notes,* 2**,** 180.

HESSA, T., KIM, H., BIHLMAIER, K., LUNDIN, C., BOEKEL, J., ANDERSSON, H., NILSSON, I., WHITE, S. H. & VON HEIJNE, G. 2005. Recognition of transmembrane helices by the endoplasmic reticulum translocon. *Nature,* 433**,** 377-81.

HIRAKI, Y., INOUE, H., IYAMA, K., KAMIZONO, A., OCHIAI, M., SHUKUNAMI, C., IIJIMA, S., SUZUKI, F. & KONDO, J. 1997. Identification of chondromodulin I as a novel endothelial cell growth inhibitor. Purification and its localization in the avascular zone of epiphyseal cartilage. *J Biol Chem,* 272**,** 32419-26.

HOSIA, W., JOHANSSON, J. & GRIFFITHS, W. J. 2002. Hydrogen/deuterium exchange and aggregation of a polyvaline and a polyleucine alpha-helix investigated by matrix-assisted laser desorption ionization mass spectrometry. *Mol Cell Proteomics,* 1**,** 592-7.

HUMPHREYS, D. T., CARVER, J. A., EASTERBROOK-SMITH, S. B. & WILSON, M. R. 1999. Clusterin has chaperone-like activity similar to that of small heat shock proteins. *J Biol Chem,* 274**,** 6875-81.

INOUYE, M. 1991. Intramolecular chaperone: the role of the pro-peptide in protein folding. *Enzyme,* 45**,** 314-21.

JARRETT, J. T. & LANSBURY, P. T., JR. 1993. Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? *Cell,* 73**,** 1055-8.

JOHANSSON, J., NERELIUS, C., WILLANDER, H. & PRESTO, J. 2010

Conformational preferences of non-polar amino acid residues: an additional factor in amyloid formation. *Biochem Biophys Res Commun,* 402**,** 515-8.

JOHANSSON, J., NILSSON, G., STROMBERG, R., ROBERTSON, B., JORNVALL, H. & CURSTEDT, T. 1995. Secondary structure and biophysical activity of synthetic analogues of the pulmonary surfactant polypeptide SP-C. *Biochem J,* 307 (Pt 2)**,** 535-41.

JOHANSSON, J., SZYPERSKI, T., CURSTEDT, T. & WUTHRICH, K. 1994. The NMR structure of the pulmonary surfactant-associated polypeptide SP-C in an apolar solvent contains a valyl-rich alpha-helix. *Biochemistry,* 33**,** 6015-23.

JOHNSON, A. L., BRAIDOTTI, P., PIETRA, G. G., RUSSO, S. J., KABORE, A., WANG, W. J. & BEERS, M. F. 2001. Post-translational processing of surfactant protein-C proprotein: targeting motifs in the NH(2)-terminal flanking domain are cleaved in late compartments. *Am J Respir Cell Mol Biol,* 24**,** 253-63.

KALLBERG, Y., GUSTAFSSON, M., PERSSON, B., THYBERG, J. & JOHANSSON, J. 2001. Prediction of amyloid fibril-forming proteins. *J Biol Chem,* 276**,** 12945-50.

KELLER, A., EISTETTER, H. R., VOSS, T. & SCHAFER, K. P. 1991. The pulmonary surfactant protein C (SP-C) precursor is a type II transmembrane protein. *Biochem J,* 277 (Pt 2)**,** 493-9.

KIM, J., MILLER, V. M., LEVITES, Y., WEST, K. J., ZWIZINSKI, C. W., MOORE, B. D., TROENDLE, F. J., BANN, M.,

VERBEECK, C., PRICE, R. W., SMITHSON, L., SONODA, L., WAGG, K., RANGACHARI, V., ZOU, F., YOUNKIN, S. G., GRAFF-RADFORD, N., DICKSON, D., ROSENBERRY, T. & GOLDE, T. E. 2008. BRI2 (ITM2b) inhibits Abeta deposition in vivo. *J Neurosci,* 28**,** 6030-6.

KIM, S. H., CREEMERS, J. W., CHU, S., THINAKARAN, G. & SISODIA, S. S. 2002. Proteolytic processing of familial British dementia-associated BRI variants: evidence for enhanced intracellular accumulation of amyloidogenic peptides. *J Biol Chem,* 277**,** 1872-7.

KIM, S. H., WANG, R., GORDON, D. J., BASS, J., STEINER, D. F., LYNN, D. G., THINAKARAN, G., MEREDITH, S. C. & SISODIA, S. S. 1999. Furin mediates enhanced production of fibrillogenic ABri peptides in familial British dementia. *Nat Neurosci,* 2**,** 984-8.

KIRCHNER, J. & BEVAN, M. J. 1999. ITM2A is induced during thymocyte selection and T cell activation and causes downregulation of CD8 when overexpressed in CD4(+)CD8(+) double positive thymocytes. *J Exp Med,* 190**,** 217-28.

KOTA, J. & LJUNGDAHL, P. O. 2005. Specialized membrane-localized chaperones prevent aggregation of polytopic proteins in the ER. *J Cell Biol,* 168**,** 79-88.

LAWSON, W. E., CROSSNO, P. F., POLOSUKHIN, V. V., ROLDAN, J., CHENG, D. S., LANE, K. B., BLACKWELL, T. R., XU, C., MARKIN, C., WARE, L. B., MILLER, G. G., LOYD, J. E. & BLACKWELL, T. S. 2008. Endoplasmic reticulum stress in alveolar epithelial cells is prominent in IPF: association with altered surfactant protein processing and herpesvirus infection. *Am J Physiol Lung Cell Mol Physiol,* 294**,** L1119-26.

LAWSON, W. E., POLOSUKHIN, V. V., STATHOPOULOS, G. T., ZOIA, O., HAN, W., LANE, K. B., LI, B., DONNELLY, E. F., HOLBURN, G. E., LEWIS, K. G., COLLINS, R. D., HULL, W. M., GLASSER, S. W., WHITSETT, J. A. & BLACKWELL, T. S. 2005. Increased and prolonged pulmonary fibrosis in surfactant protein C-deficient mice following intratracheal bleomycin. *Am J Pathol,* 167**,** 1267-77.

LI, J., HOSIA, W., HAMVAS, A., THYBERG, J., JORNVALL, H., WEAVER, T. E. & JOHANSSON, J. 2004. The N-terminal propeptide of lung surfactant protein C is necessary for biosynthesis and prevents unfolding of a metastable alpha-helix. *J Mol Biol,* 338**,** 857-62.

MAGUIRE, J. A., MULUGETA, S. & BEERS, M. F. 2010. Endoplasmic Reticulum Stress Induced by Surfactant Protein C BRICHOS Mutants Promotes Proinflammatory Signaling by Epithelial Cells. *Am J Respir Cell Mol Biol,* 44**,** 404-14.

MAKIN, O. S. & SERPELL, L. C. 2005. Structures for amyloid fibrils. *FEBS J,* 272**,** 5950-61.

MARTIN, L., FLUHRER, R., REISS, K., KREMMER, E., SAFTIG, P. & HAASS, C. 2008. Regulated intramembrane proteolysis of Bri2 (Itm2b) by ADAM10 and SPPL2a/SPPL2b. *J Biol Chem,* 283**,** 1644-52.

MARTIN, T. E., POWELL, C. T., WANG, Z., BHATTACHARYYA, S., WALSH-REITZ, M. M., AGARWAL, K. & TOBACK, F. G. 2003. A novel mitogenic protein that is highly expressed in cells of the gastric antrum mucosa. *Am J Physiol Gastrointest Liver Physiol,* 285**,** G332-43.

MATSUDA, S., MATSUDA, Y. & D'ADAMIO, L. 2009a. BRI3 inhibits amyloid precursor protein processing in a mechanistically distinct manner from its homologue dementia gene BRI2. *J Biol Chem,* 284**,** 15815-25.

MATSUDA, S., MATSUDA, Y., SNAPP, E. L. & D'ADAMIO, L. 2009b. Maturation of BRI2 generates a specific inhibitor that reduces APP processing at the plasma membrane and in endocytic vesicles. *Neurobiol Aging.*

MENHENIOTT, T. R., PETERSON, A. J., O'CONNOR, L., LEE, K. S., KALANTZIS, A., KONDOVA, I., BONTROP, R. E., BELL, K. M. & GIRAUD, A. S. 2010. A novel gastrokine, Gkn3, marks gastric atrophy and shows evidence of adaptive gene loss in humans. *Gastroenterology,* 138**,** 1823-35.

MINGARRO, I., NILSSON, I., WHITLEY, P. & VON HEIJNE, G. 2000. Different conformations of nascent polypeptides during translocation across the ER membrane. *BMC Cell Biol,* 1**,** 3.

MUCHOWSKI, P. J. & WACKER, J. L. 2005. Modulation of neurodegeneration by molecular chaperones. *Nat Rev Neurosci,* 6**,** 11-22.

MULUGETA, S., MAGUIRE, J. A., NEWITT, J. L., RUSSO, S. J., KOTORASHVILI, A. & BEERS, M. F. 2007. Misfolded BRICHOS SP-C mutant proteins induce apoptosis via caspase-4- and cytochrome c-related mechanisms. *Am J Physiol Lung Cell Mol Physiol,* 293**,** L720-9.

NAKAGAMI, Y., NISHIMURA, S., MURASUGI, T., KANEKO, I., MEGURO, M., MARUMOTO, S., KOGEN, H., KOYAMA, K. & ODA, T. 2002. A novel beta-sheet breaker, RS-0406, reverses amyloid beta-induced cytotoxicity and impairment of long-term potentiation in vitro. *Br J Pharmacol,* 137**,** 676-82.

NERELIUS, C., GUSTAFSSON, M., NORDLING, K., LARSSON, A. & JOHANSSON, J. 2009. Anti-amyloid activity of the C-terminal domain of proSP-C against amyloid beta-peptide and medin. *Biochemistry,* 48**,** 3778-86.

NILSSON, G., GUSTAFSSON, M., VANDENBUSSCHE, G., VELDHUIZEN, E., GRIFFITHS, W. J., SJOVALL, J., HAAGSMAN, H. P., RUYSSCHAERT, J. M., ROBERTSON, B., CURSTEDT, T. & JOHANSSON, J. 1998. Synthetic peptide-containing surfactants--evaluation of transmembrane versus amphipathic helices and surfactant protein C poly-valyl to poly-leucyl substitution. *Eur J Biochem,* 255**,** 116-24.

NOGEE, L. M., DUNBAR, A. E., 3RD, WERT, S., ASKIN, F., HAMVAS, A. & WHITSETT, J. A. 2002. Mutations in the surfactant protein C gene associated with interstitial lung disease. *Chest,* 121**,** 20S-21S.

NOGEE, L. M., DUNBAR, A. E., 3RD, WERT, S. E., ASKIN, F., HAMVAS, A. & WHITSETT, J. A. 2001. A mutation in the surfactant protein C gene associated with familial interstitial lung disease. *N Engl J Med,* 344**,** 573-9.

OIEN, K. A., MCGREGOR, F., BUTLER, S., FERRIER, R. K., DOWNIE, I., BRYCE, S., BURNS, S. & KEITH, W. N. 2004. Gastrokine 1 is abundantly and specifically expressed in superficial gastric epithelium, down-regulated in gastric carcinoma, and shows high evolutionary conservation. *J Pathol,* 203**,** 789-97.

OTTO, W. R., PATEL, K., MCKINNELL, I., EVANS, M. D., LEE, C. Y., FRITH, D., HANRAHAN, S., BLIGHT, K., BLIN, N., KAYADEMIR, T., POULSOM, R., JEFFERY, R., HUNT, T., WRIGHT, N. A., MCGREGOR, F. & OIEN, K. A. 2006. Identification of blottin: a novel gastric trefoil factor family-2 binding protein. *Proteomics,* 6**,** 4235-45.

PALMBLAD, M., JOHANSSON, J., ROBERTSON, B. & CURSTEDT, T. 1999. Biophysical activity of an artificial surfactant containing an analogue of surfactant protein (SP)-C and native SP-B. *Biochem J,* 339 (Pt 2)**,** 381-6.

PENG, S., FITZEN, M., JORNVALL, H. & JOHANSSON, J. 2010. The extracellular domain of Bri2 (ITM2B) binds the ABri peptide (1-23) and amyloid beta-peptide (Abeta1-40): Implications for Bri2 effects on processing of amyloid precursor protein and Abeta aggregation. *Biochem Biophys Res Commun,* 393**,** 356-61.

PERSSON, B. & ARGOS, P. 1994. Prediction of transmembrane segments in proteins utilising multiple sequence alignments. *J Mol Biol,* 237**,** 182-92.

PLOTKIN, S. S. & ONUCHIC, J. N. 2002. Understanding protein folding with energy landscape theory. Part I: Basic concepts. *Q Rev Biophys,* 35**,** 111-67.

POLSHAKOV, V. I., WILLIAMS, M. A., GARGARO, A. R., FRENKIEL, T. A., WESTLEY, B. R., CHADWICK, M. P., MAY, F. E. & FEENEY, J. 1997. High-resolution solution

structure of human pNR-2/pS2: a single trefoil motif protein. *J Mol Biol,* 267**,** 418-32.
RADFORD, S. E. 2000. Protein folding: progress made and promises ahead. *Trends Biochem Sci,* 25**,** 611-8.
ROSTAGNO, A., TOMIDOKORO, Y., LASHLEY, T., NG, D., PLANT, G., HOLTON, J., FRANGIONE, B., REVESZ, T. & GHISO, J. 2005. Chromosome 13 dementias. *Cell Mol Life Sci,* 62**,** 1814-25.
SANCHEZ-PULIDO, L., DEVOS, D. & VALENCIA, A. 2002. BRICHOS: a conserved domain in proteins associated with dementia, respiratory distress and cancer. *Trends Biochem Sci,* 27**,** 329-32.
SANDBERG, A., LUHESHI, L. M., SOLLVANDER, S., PEREIRA DE BARROS, T., MACAO, B., KNOWLES, T. P., BIVERSTAL, H., LENDEL, C., EKHOLM-PETTERSON, F., DUBNOVITSKY, A., LANNFELT, L., DOBSON, C. M. & HARD, T. 2010. Stabilization of neurotoxic Alzheimer amyloid-beta oligomers by protein engineering. *Proc Natl Acad Sci U S A,* 107**,** 15595-600.
SHUKUNAMI, C., OSHIMA, Y. & HIRAKI, Y. 2001. Molecular cloning of tenomodulin, a novel chondromodulin-I related gene. *Biochem Biophys Res Commun,* 280**,** 1323-7.
SIPE, J. D., BENSON, M. D., BUXBAUM, J. N., IKEDA, S., MERLINI, G., SARAIVA, M. J. & WESTERMARK, P. 2010. Amyloid fibril protein nomenclature: 2010 recommendations from the nomenclature committee of the International Society of Amyloidosis. *Amyloid,* 17**,** 101-4.
SISODIA, S. S. 1992. Beta-amyloid precursor protein cleavage by a membrane-bound protease. *Proc Natl Acad Sci U S A,* 89**,** 6075-9.
SOLARIN, K. O., WANG, W. J. & BEERS, M. F. 2001. Synthesis and post-translational processing of surfactant protein C. *Pediatr Pathol Mol Med,* 20**,** 471-500.
STEVENS, P. A., PETTENAZZO, A., BRASCH, F., MULUGETA, S., BARITUSSIO, A., OCHS, M., MORRISON, L., RUSSO, S. J. & BEERS, M. F. 2005. Nonspecific interstitial pneumonia, alveolar proteinosis, and abnormal proprotein trafficking resulting from a spontaneous mutation in the surfactant protein C gene. *Pediatr Res,* 57**,** 89-98.
SZYPERSKI, T., VANDENBUSSCHE, G., CURSTEDT, T., RUYSSCHAERT, J. M., WUTHRICH, K. & JOHANSSON, J. 1998. Pulmonary surfactant-associated polypeptide C in a mixed organic solvent transforms from a monomeric alpha-helical state into insoluble beta-sheet aggregates. *Protein Sci,* 7**,** 2533-40.
THOMAS, A. Q., LANE, K., PHILLIPS, J., 3RD, PRINCE, M., MARKIN, C., SPEER, M., SCHWARTZ, D. A., GADDIPATI,

R., MARNEY, A., JOHNSON, J., ROBERTS, R., HAINES, J., STAHLMAN, M. & LOYD, J. E. 2002. Heterozygosity for a surfactant protein C gene mutation associated with usual interstitial pneumonitis and cellular nonspecific interstitial pneumonitis in one kindred. *Am J Respir Crit Care Med,* 165**,** 1322-8.

TOMIDOKORO, Y., LASHLEY, T., ROSTAGNO, A., NEUBERT, T. A., BOJSEN-MOLLER, M., BRAENDGAARD, H., PLANT, G., HOLTON, J., FRANGIONE, B., REVESZ, T. & GHISO, J. 2005. Familial Danish dementia: co-existence of Danish and Alzheimer amyloid subunits (ADan AND A{beta}) in the absence of compact plaques. *J Biol Chem,* 280**,** 36883-94.

TREDANO, M., GRIESE, M., BRASCH, F., SCHUMACHER, S., DE BLIC, J., MARQUE, S., HOUDAYER, C., ELION, J., COUDERC, R. & BAHUAU, M. 2004. Mutation of SFTPC in infantile pulmonary alveolar proteinosis with or without fibrosing lung disease. *Am J Med Genet A,* 126A**,** 18-26.

WALSH, D. M., THULIN, E., MINOGUE, A. M., GUSTAVSSON, N., PANG, E., TEPLOW, D. B. & LINSE, S. 2009. A facile method for expression and purification of the Alzheimer's disease-associated amyloid beta-peptide. *FEBS J,* 276**,** 1266-81.

VAN DEN PLAS, D. & MERREGAERT, J. 2004. In vitro studies on Itm2a reveal its involvement in early stages of the chondrogenic differentiation pathway. *Biol Cell,* 96**,** 463-70.

WANG, W. J., MULUGETA, S., RUSSO, S. J. & BEERS, M. F. 2003. Deletion of exon 4 from human surfactant protein C results in aggresome formation and generation of a dominant negative. *J Cell Sci,* 116**,** 683-92.

WANG, W. J., RUSSO, S. J., MULUGETA, S. & BEERS, M. F. 2002. Biosynthesis of surfactant protein C (SP-C). Sorting of SP-C proprotein involves homomeric association via a signal anchor domain. *J Biol Chem,* 277**,** 19929-37.

VASSAR, R., BENNETT, B. D., BABU-KHAN, S., KAHN, S., MENDIAZ, E. A., DENIS, P., TEPLOW, D. B., ROSS, S., AMARANTE, P., LOELOFF, R., LUO, Y., FISHER, S., FULLER, J., EDENSON, S., LILE, J., JAROSINSKI, M. A., BIERE, A. L., CURRAN, E., BURGESS, T., LOUIS, J. C., COLLINS, F., TREANOR, J., ROGERS, G. & CITRON, M. 1999. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science,* 286**,** 735-41.

WESTLEY, B. R., GRIFFIN, S. M. & MAY, F. E. 2005. Interaction between TFF1, a gastric tumor suppressor trefoil protein, and TFIZ1, a brichos domain-containing protein with homology to SP-C. *Biochemistry,* 44**,** 7967-75.

WHITSETT, J. A. & WEAVER, T. E. 2002. Hydrophobic surfactant proteins in lung function and disease. *N Engl J Med,* 347**,** 2141-8.

WICKHAM, L., BENJANNET, S., MARCINKIEWICZ, E., CHRETIEN, M. & SEIDAH, N. G. 2005. Beta-amyloid protein converting enzyme 1 and brain-specific type II membrane protein BRI3: binding partners processed by furin. *J Neurochem,* 92**,** 93-102.

VIDAL, R., CALERO, M., REVESZ, T., PLANT, G., GHISO, J. & FRANGIONE, B. 2001. Sequence, genomic structure and tissue expression of Human BRI3, a member of the BRI gene family. *Gene,* 266**,** 95-102.

VIDAL, R., FRANGIONE, B., ROSTAGNO, A., MEAD, S., REVESZ, T., PLANT, G. & GHISO, J. 1999. A stop-codon mutation in the BRI gene associated with familial British dementia. *Nature,* 399**,** 776-81.

VIDAL, R., REVESZ, T., ROSTAGNO, A., KIM, E., HOLTON, J. L., BEK, T., BOJSEN-MOLLER, M., BRAENDGAARD, H., PLANT, G., GHISO, J. & FRANGIONE, B. 2000. A decamer duplication in the 3' region of the BRI gene originates an amyloid peptide that is associated with dementia in a Danish kindred. *Proc Natl Acad Sci U S A,* 97**,** 4920-5.

WOISCHNIK, M., SPARR, C., KERN, S., THURM, T., HECTOR, A., HARTL, D., LIEBISCH, G., MULUGETA, S., BEERS, M. F., SCHMITZ, G. & GRIESE, M. 2010. A non-BRICHOS surfactant protein c mutation disrupts epithelial cell function and intercellular signaling. *BMC Cell Biol,* 11**,** 88.

VORBROKER, D. K., DEY, C., WEAVER, T. E. & WHITSETT, J. A. 1992. Surfactant protein C precursor is palmitoylated and associates with subcellular membranes. *Biochim Biophys Acta,* 1105**,** 161-9.

VORBROKER, D. K., VOORHOUT, W. F., WEAVER, T. E. & WHITSETT, J. A. 1995. Posttranslational processing of surfactant protein C in rat type II cells. *Am J Physiol,* 269**,** L727-33.

YERBURY, J. J., KUMITA, J. R., MEEHAN, S., DOBSON, C. M. & WILSON, M. R. 2009. alpha2-Macroglobulin and haptoglobin suppress amyloid formation by interacting with prefibrillar protein species. *J Biol Chem,* 284**,** 4246-54.

YERBURY, J. J., POON, S., MEEHAN, S., THOMPSON, B., KUMITA, J. R., DOBSON, C. M. & WILSON, M. R. 2007. The extracellular chaperone clusterin influences amyloid formation and toxicity by interacting with prefibrillar structures. *FASEB J,* 21**,** 2312-22.

YOUNKIN, S. G. 1995. Evidence that A beta 42 is the real culprit in Alzheimer's disease. *Ann Neurol,* 37**,** 287-8.

ZHANG, Y. W., THOMPSON, R., ZHANG, H. & XU, H. 2011. APP processing in Alzheimer's disease. *Mol Brain,* 4**,** 3.

5 Acknowledgements

Även om det bara är mitt namn som står på framsidan så hade den här avhandlingen aldrig skrivits utan hjälp och stöd från många kollegor och vänner. Särskilt stort tack vill jag rikta till:

Min handledare, **Janne**, för att jag fick börja som doktorand hos dig, och för ditt otroliga intresse och engagemang för forskning, för att du alltid kan hitta något positivt i alla resultat och för alla mångkamper på Grönan och grupputflykter till Dalarna. Den här boken hade uppenbarligen aldrig skrivits utan dig! Tack!

Min bihandledare, **Tommy Linné**, för att du introducerade mig för den spännande prion forskningen.

To all my **co-authors** and **collaborators**, thanks for nice papers, fruitful discussions about science and for sharing your scientific knowledge with me.

Till alla gamla och nya medlemmar i **JJ-gruppen**: **Kerstin**, för alla diskussioner om allt och inget, från pH i buffertar till hur man gör för att sälja saker på blocket och för att du har delat med dig av all din kunskap. Du har varit den bästa rumskompisen man kan tänka sig! **Jenny**, det har varit så kul att få dela BRICHOS forskningen med dig, och tack för alla diskussioner om forskning och familjeliv och alla roliga resor vi har åkt på tillsammans. Flugmannen **Erik**, tack för allt gott fikabröd, och hjälp med datorn, lycka till med flugorna i Huddinge! **Anna R,** för att du alltid är så positiv och för alla stunder tillsammans med dig och din familj. **Stefan**, för att du alltid är så omtänksam och positiv och du, Hanna och barnen har blivit våra väldigt kära vänner. **My,** för din aldrig sinande kunskap om

proteinrening och nya idéer om forskning, och för ovärderlig hjälp med endnote formatteringen. **Ingrid,** vi tyckte att vi hade ordning i labbet tills du kom! tack för att du håller efter oss och för dina fantastiska bakverk. **Ronnie,** min nya ”roomie”, som har ett protokoll för allt, och som hittar alla möjliga och omöjliga buffertar i kylrummet, det kommer att kännas tomt utan våra tidiga fikastunder. **Mona**, du är alltid så glad och tack för din skönsång på labbet! **Sara**, det har varit tomt utan dig det här halvåret, tack för alla trevliga fikastunder i röda soffan. **Anton**, för att någon annan är på min sida mot alla ajfån användare, och för alla diskussioner om precis allt. **Charlotte**, det har varit tomt utan dig och jag har saknat våra röda soffan-diskussioner om forskning och mera världsliga ting**.** Lycka till i sommar! **Diana,** supertrevligt att få lära känna dig även om du inte blev kvar så länge, lycka till uppe i norr. **Urmi** and **Siwei**, for bringing some culture into our group and for all your nice cooking! I have missed you. **Anna S,** tack för att du körde mina prover i ultracentrifugen och fick mig att inse att centrifuger bara är lite farliga ☺, **Jing, Margareta, Maria, Magnus, Andreas, Waltteri** and **Anna P**, it has been a pleasure working with you all and thanks for all the good times both in and outside the lab.

Lotta, det har varit jättekul att labba med dig, både i och utanför prionlabbet, och tack för alla trevliga stunder tillsammans med dig och Minna. **Gelareh,** för ditt smittande skratt, otroliga energi och allt arbete som du har lagt ner på BRICHOS strukturen och för vår lärorika tid tillsammans på SGC.

Piotr, vår livlina, finns det något som du inte klarar av? Tack för all hjälp med allt det tekniska, datorn, Äktan och fraktiossamlaren.

Louise, det har varit så tryggt att ha någon med sig på den spännande men jobbiga resan mot en färdig bok. Tack för all hjälp med bilderna och alla pratstunder om allt och inget! Snart är det din tur.

Till alla gamla och nya medlemmar i **GP-, SE- och IB-gruppen**: **Gunnar, Sara, Ida, Elin, Gabriella, Helena, Fabio, Gianni, Osama, Ignacio, Svetlana, Mirjana, Magnus Å, Elena, Staffan, Liya, Elena S, Hanan, Ren, Jay, Kieran**, **Ashraf, Gunnar F, Rahma, Ingmar och Elke** thanks for all the nice chats in the red sofa and for making B9:4 the best corridor to work in at BMC!

Till alla på **SGC**, för att ni tog emot Gelareh och mig med öppna armar. Det var verkligen lärorikt även om det inte riktigt var så lätt som vi trodde att få fram strukturen!

Det ”gamla gänget”, **Jenny H-M, Frida, Cecilia, Anders,** det var hos er allt började en gång i tiden. Tack för alla roliga fester, trevliga middagar och träningssällskap!

Till alla kompisar utanför jobbet, tack för alla skidresor, semestrar, fester och middagar och för att ni finns där för mig och min familj!

Sist men inte minst, min familj!

Till min bonusfamilj, **Anna, Roger, Gustav, Hedvig, Stella, Karin, Leif** och **familjen Rosendahl.** Tack för att ni finns där och för alla mysiga stunder tillsammans. För att ni alltid frågar hur det går även om ni inte riktigt förstår vad jag forskar på.

Åsa, du finns alltid där när man behöver prata av sig och för att du förstår hur det är att doktorera, för att du och **Micke** alltid ställer upp. Det har varit väldigt tomt i Uppsala utan er.

Mamma och **pappa**, ni är de bästa föräldrar som finns! Tack för allt ert stöd, även om det är långt mellan oss så finns ni bara ett telefonsamtal bort. Tack!

Alva och **Ville,** mina älskade små ligister som har skänkt mig så mycket glädje och några gråa hårstrån. Varje dag med er är ett nytt äventyr och ni är mitt allt. Puss på er!

Fredrik, du finns alltid vid min sida och du vill alltid mitt och barnens bästa. För dig är ingenting omöjligt. Jag älskar dig!