CHARACTERIZATION OF THE ALZHEIMER'S DISEASE-ASSOCIATED CLAC PROTEIN

Linda Söderberg

Stockholm 2005

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Karolinska Institutet
Neurotec

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CONTENTS

ABSTRACT

Alzheimer's disease (AD) is the most prevalent form of dementia in elderly people and is neuropathologically characterized by the presence of extracellular senile plaques (SPs) in the brain. SPs are mainly composed of the amyloid β-peptide (Aβ), proteolytically derived from the amyloid β-precursor protein by sequential cleavages performed by β- and γ secretases, to generate Aβ species of 40 and 42 amino acids, respectively. The mechanism underlying the formation of SPs is largely unknown but it is possible that the less dense Aβ deposits, referred to as diffuse plaques, represent precursors to the SPs that occurs in the brain at a later stage of the disease. In addition to Aβ, a number of other non-Aβ components have been found to co-deposit with SPs. Many studies performed *in vitro* and *in vivo* on these so-called plaque-associated proteins suggest that they are able to modulate Aβ aggregation, either by facilitating or preventing Aβ fibril growth. An increased plaque load in the brain may also be caused by Aβ-binding components that increase the persistence of plaque by affecting the clearance. Thus, further characterization of these proteins and their impact on Aβ aggregation is imperative for understanding the events underlying formation of amyloid deposits *in vivo*.

The work presented in this thesis has focused on one of these plaque-associated proteins, the collagenous Alzheimer amyloid plaque component (CLAC) and its interaction with Aβ. CLAC is generated through furin cleavage of the precursor protein CLAC-P/collagen XXV, a novel transmembrane collagen. In **paper I** we found that CLAC was the antigen causing the AMY immunoreactivity, which is co-localized with SPs in AD brain. A system for the expression and purification of recombinant CLAC in a mammalian cell line was devised in **paper II**, which enabled us to study the *in vitro* properties of CLAC. We showed that CLAC displays features characteristic of a collagen protein, e.g. it forms a partly protease-resistant triple-helical structure, exhibits an intermediate affinity for heparin and is glycosylated. In order to study the molecular interactions between CLAC and Aβ *in vitro*, we developed a solid-phase binding immunoassay. CLAC was found to bind aggregated Aβ in a concentration-dependent manner and binding was impaired by increasing salt concentrations, suggesting a partly ionic dependent CLAC/Aβ interaction. The LIKRRLIK sequence within non-collagenous domain 2 of CLAC was identified as an Aβ-binding motif. Deletion or substitution of this motif generated CLAC variants with diminished Aβ-binding affinity. In **paper III**, sequences in Aβ involved in CLAC-binding were examined. The central region of Aβ was shown to be necessary and sufficient for binding to CLAC and the binding correlated better with the aggregation state of the peptide, than with the primary sequence. This suggests that CLAC has low sequence specificity and is in agreement with observations presented in **paper II**, which show that CLAC is able to bind other fibrils formed from non-amyloid-β component and amylin.

Understanding the role of CLAC/Aβ interaction in AD is essential for future research aiming at interacting with the CLAC/Aβ binding. In **paper III** the effect of CLAC on Aβ fibril elongation was examined using surface plasmon resonance spectroscopy. Treatment of Aβ fibrils with CLAC reduced the rate of Aβ fibril elongation by 20 to 30%. The effect of CLAC on Aβ fibrils was further studied in **paper IV** using assays based on turbidity, Thioflavin T binding, sedimentation analysis and electron microscopy. Incubation of preformed fibrils with CLAC led to the formation of larger aggregates compared to non CLAC-treated samples. Finally, we investigated whether the CLAC-induced fibrils could be of some biological relevance by a protease resistance assay. Aβ fibrils incubated with CLAC were more resistant to proteolytic degradation and it is possible that CLAC may act in a similar way *in vivo*. We suggest that CLAC effects AD pathogenesis by binding and assembling Aβ aggregates and thereby altering the turnover and persistence of SPs in AD brain.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numbers:

I **Linda Söderberg**, Victoria Zhukareva, Nenad Bogdanovic, Tadafumi Hashimoto, Bengt Winblad, Takeshi Iwatsubo, Virginia M. Y. Lee, John Q. Trojanowski, Jan Näslund

Molecular identification of AMY, an Alzheimer disease amyloid-associated protein

Journal of Neuropathology and Experimental Neurology (2003) **62**, 1108-1117

II **Linda Söderberg**, Hiroyoshi Kakuyama, Anna Möller, Akira Ito, Bengt Winblad, Lars O. Tjernberg, Jan Näslund

Characterization of the Alzheimer's disease-associated CLAC protein and identification of an amyloid β-peptide-binding site

The Journal of Biological Chemistry (2005) **280,** 1007-1015

III Hiroyoshi Kakuyama, **Linda Söderberg**, Kazuhiko Horigome, Camilla Dahlqvist, Bengt Winblad, Jan Näslund, Lars O. Tjernberg

CLAC binds to aggregated Aβ and Aβ fragments, and attenuates fibril elongation

Submitted (2005)

IV **Linda Söderberg***, Camilla Dahlqvist*, Hiroyoshi Kakuyama, Johan Thyberg, Akira Ito, Bengt Winblad, Jan Näslund, Lars O. Tjernberg

CLAC assembles amyloid fibrils into protease resistant aggregates

FEBS Journal in press (2005)

* These authors contributed equally to this work

ABBREVIATIONS

Aβ	Amyloid β-peptide
AChE	Acetylcholinesterase
ACT	α1-antichymotrypsin
AD	Alzheimer's disease
ADDLs	Aβ-derived diffusible ligands
ApoE	Apolipoprotein E
APP	Amyloid β-precursor protein
CD	Circular dichroism
CLAC	Collagenous Alzheimer amyloid plaque component
CLAC-P	Collagenous Alzheimer amyloid plaque component precursor/collagen XXV
COL	Collagenous
CSF	Cerebrospinal fluid
DS	Down's syndrome
ECM	Extracellular matrix
EM	Electron microscopy
FA	Formic acid
FAD	Familial Alzheimer's disease
GAG	Glycosaminoglycan
HEK	Human embryonic kidney
HSPG	Heparan sulfate proteoglycan
mAbs	Monoclonal antibodies
NAC	Non-amyloid-β component
NC	Non-collagenous
NFT	Neurofibrillary tangle
PHF	Paired helical filament
PS	Presenilin
SAD	Sporadic AD
SAP	Serum amyloid P
SDS	Sodium dodecyl sulphate
SP	Senile plaque
SPR	Surface plasmon resonance
ThT	Thioflavin T
Wt	Wild type

INTRODUCTION

ALZHEIMER'S DISEASE

General Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder and the major cause of dementia in elderly people, accounting for 60-70% of all dementia cases [1]. Clinically, the disease is characterized by progressive memory loss and cognitive impairment [2]. Neuropathologically, the characteristic features are neurofibrillary tangles (NFTs), senile plaques (SPs) and brain atrophy due to the extensive neuronal loss [3]. The duration of the disease varies between 5-15 years, starting with short-term memory loss followed by progressive cognitive dysfunction. The cause of death is usually associated with secondary illnesses such as pneumonia or infections. The major risk factors for AD are aging and a family history of dementia. The disease is classified as sporadic AD (SAD) without a clear inheritance pattern and familial AD (FAD), which accounts for about 10% of all AD cases [4]. The prevalence of AD is around 1% at age 65 and 20% at age 80 years [5]. Mutations in the *APP* gene on chromosome 21, or the presenilin genes 1 (*PS1*) and 2 (*PS2*) on chromosome 14 and 1, respectively, greatly increase the risk of developing FAD. The ε4 isoform of the gene encoding apolipoprotein E (apoE) on chromosome 19 is a major genetic risk factor for developing SAD [6, 7]. In addition, polymorphisms in the gene for α2-macroglobulin gene on chromosome 12 are associated with the disease [8, 9].

Although the knowledge about AD has increased rapidly the last ten years there is no cure for the disease. The number of people affected has been estimated to triple in the next few decades due to the longer lifespan [10]. This will be accompanied by an enormous increase in AD related costs for society. The drugs available today only relive symptoms either by raising levels of acetylcholine or reducing the inflammatory process seen in the proximity of the SPs associated with AD. Intense research is therefore focused on the development of disease-modifying drugs, which treat or prevent the disease progression. New paradigms for treatment include anti-amyloid strategies, for instance immunotherapy-based approaches that will prevent the build-up of amyloid present in the SPs [11] and drugs that inhibit Aβ polymerization [12, 13].

Neuropathology of Alzheimer's Disease

In 1907, the German physician Alois Alzheimer described the first case of what was later to be called Alzheimer's disease, from postmortem examination of a 51-year old woman named Auguste D [14]. Two lesions in particular were described which now are the hallmarks of the disease: NFTs and SPs (Figure 1). Another characteristic feature of the disease is the extensive amyloid deposits in the vessel walls of the brain. SPs are extracellular deposits mainly composed of fibrils formed from the 40-42 amino acid amyloid β-peptide (Aβ) [15, 16]. NFTs are intracellular helical filaments composed of hyperphosphorylated tau, a microtubule-binding protein [17]. NFTs are also present in other brain disorders such as the

neurodegenerative tauopathies, including Pick's disease, progressive supranuclear palsy and hereditary frontotemperal dementia and parkinsonism linked to chromosome 17 (FTDP-17) [18]. In AD the earliest affected regions are the neocortical association areas and limbic structures, especially the hippocampus, enthorinal cortex and amygdala [19]. At later stages of the disease, the primary motor and sensory cortex are affected by AD pathology.

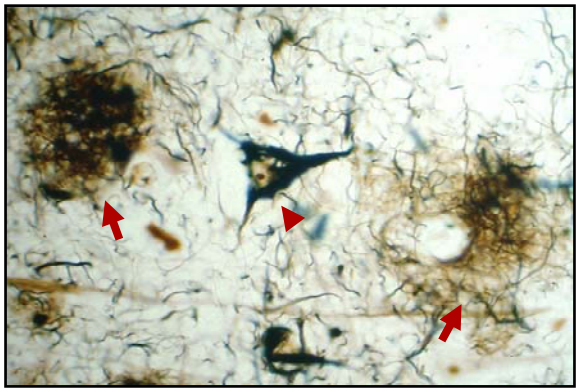

Figure 1: The main lesions of AD. Extracellular senile plaques (arrows) consist of an amyloid core surrounded by numerous neurites. Intracellular neurofibrillary tangle (arrowhead). Bielschowsky silver staining. Photo by Nenad Bogdanovic.

Plaques are divided into two major categories based on their different morphologies; neuritic and diffuse plaques. The diffuse plaques, also called primitive or pre-amyloid deposits, are mainly composed of amorphous Aβ42 and are not surrounded by dystrophic neurites [20]. They are detected by immunohistochemical methods but not by the classical amyloid dyes, Congo red and thioflavin S, indicating a lack of β-sheet structure. The diffuse plaques are thought to be precursor lesions to the neuritic plaques. This notion is partly based on studies in transgenic mice where the diffuse plaques precede the appearance of neuritic plaques. Additional support for this hypothesis comes from Down syndrome (DS) individuals, who develop AD pathology early in life and display diffuse plaques before development of neuritic plaques [21].

The neuritic plaques, commonly called mature or senile plaques, are associated with the classical pathology of AD and are found in large numbers at later stages of the disease process. They are composed of fibrillar Aβ in β-sheet conformation and are therefore easily detected by the amyloid-binding dyes. The majority of Aβ in the plaque cores end at amino acid 42, which is the first species deposited in AD brains [21, 22]. Activated microglia and reactive astrocytes are associated with most of these plaques, usually in the vicinity of the dense amyloid core [23, 24]. Interaction of these cells with Aβ leads to release of potentially toxic molecules, including pro-inflammatory cytokines and reactive oxygen species. These cells also produce a number of other plaque-associated proteins such as apoE and J, and α2-macroglobulin. Plaques do not only occur in these two forms (diffuse and neuritic) but rather as a continuum from diffuse less organized deposits to the dense fibrillar Aβ deposits. The different plaque types have been classified into several subgroups with varying degrees of glial alterations [25]. A rather novel type of plaques, cotton-wool plaques, were for the first

time described in a Finnish pedigree with a variant of AD characterized by spasticity of the lower limbs [26]. The cotton-wool plaques are large, roundish and resemble the diffuse plaques since the major component is Aβ42. They lack an amyloid core, are thioflavin S negative and no neuritic processes are involved. Similar plaques have been described in other families carrying mutations in the *PS1* gene [27]. Fleecy amyloid is another distinct type of Aβ plaque, which consists exclusively of N-terminally truncated Aβ peptides [28]. These plaques are probably intermediate deposits which eventually mature into SPs, since they are not detected in later stages of the disease. It is worth noting that some aged individuals can have large numbers of diffuse plaques without any signs of neuronal dysfunction or dementia.

The Amyloid β-Peptide

The identity of Aβ has now been known for more than two decades. Aβ was initially isolated and sequenced from meningeal vessel of AD and DS patients [15], and was later shown to be the primary component of the SPs [16]. Aβ is a cleavage product of the amyloid β-precursor protein (APP), a type I integral membrane protein expressed throughout the whole body. The *APP* gene is located on chromosome 21 and its mRNA undergoes alternative splicing to generate 8 different isoforms [29]. The 695, 751 and 770 APP isoforms dominate in the brain and APP695 is primarily expressed in neurons [30]. The Aβ sequence lies partly in the transmembrane region of APP, and the sequential cleavages performed by β- and γ-secretases release Aβ [31]. Cleavage by α-secretase instead of β-secretase precludes the formation of Aβ since this cleavage occurs within the Aβ sequence. γ-Secretase, which generates the C terminus of Aβ, is a multiprotein complex comprising four components; the presenilin proteins (PS), nicastrin [32], Aph-1 [33, 34] and Pen-2 [33]. γ-Secretase shows low sequence specificity and generates Aβ species of different length. The major species produced are Aβ40 and Aβ42, ending at Val40 and Ala42, respectively (Figure 2) but species ending at residues 38, 39 and 43 have also been detected [35, 36]. In healthy individuals, Aβ40 accounts for about 90% of the produced Aβ, and is found at higher concentration in both plasma and cerebrospinal fluid (CSF) than Aβ42 [37].

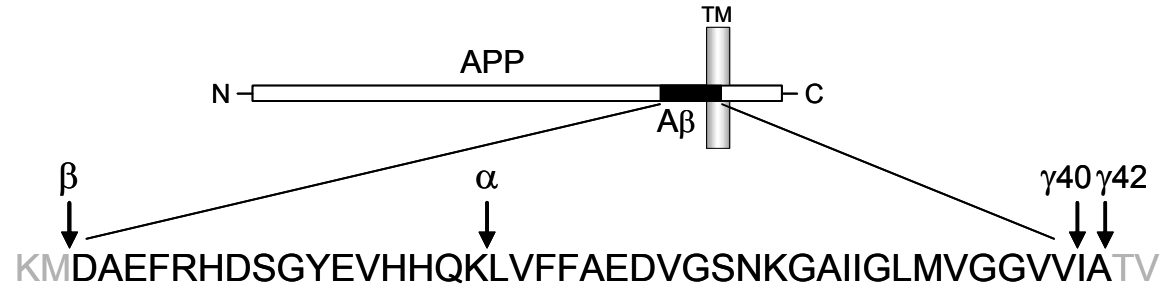

Figure 2: Schematic illustration of the APP molecule containing Aβ (black box) and the transmembrane region (TM). Cleavages performed at β- and γ-secretase sites of APP generate Aβ ending at Val40 and Ala42. Cleavages at the α-secretase site precludes the formation of Aβ.

For over ten years, the dominating hypothesis explaining the development of AD has been the "amyloid cascade hypothesis", which states that AD results from neuronal death caused by Aβ aggregation into SPs. According to this model tangle formation and inflammatory responses occur secondary to deposition of Aβ [3, 38]. The hypothesis is supported by the finding that FAD mutations lead to an increased Aβ production and/or an increased Aβ42/Aβ40 ratio that will favor the deposition of the more amyloidogenic Aβ42 species. An increased Aβ production and early AD pathology is observed also in DS patients carrying an additional copy of the *APP* gene. Further, SPs have been shown to precede tangle formation in a triple transgenic mice model overexpressing mutant APP and tau on a PS1 knock in background [39]. During the last years the "amyloid cascade hypothesis" has gradually been refined based on evidence suggesting that SPs *per se* might not be the primary mechanism underlying neurodegeneration. The form(s) of Aβ which causes injury to neurons *in vivo* has not yet been identified. The emphasis is now on soluble oligomeric forms of Aβ which correlates better with the degree of dementia than amyloid deposits [40-42]. This form of Aβ seems to be more toxic to neurons than the mature amyloid fibrils [43].

Aβ Fibril Formation

Aβ fibrils isolated from amyloid deposits in brain tissue and those formed *in vitro* have very similar morphologies and staining characteristics. The fibrils have a cross-β sheet structure, a structure in which the β-sheets run perpendicular to the fibril axis [44]. Visualization of the fibrils with electron microscopy (EM) shows that they are straight, unbranched and have an average diameter of about 10 nm and longer than 200 nm [45, 46]. X-ray diffraction patterns of the fibrils show a 4.8 Å and a 10.7 Å reflection that corresponds to the distance between the β-strands and the distance between two sheets, respectively [47, 48]. When stained with Congo red they exhibit red/green birefringence under polarized light, indicating an ordered structure. Both fibrils formed *in vivo* and *in vitro* can be stained by the fluorescent dyes thioflavin S and T, resulting in an altered fluorescence spectrum. Due to the similarities in morphology, *in vitro* systems offer a good model for studying Aβ fibril growth in order to understand the process leading to Aβ aggregation into SPs.

At concentrations in the μM range, Aβ assembles into fibrils in a nucleation-dependent process which is characterized by a slow nucleation phase and a fast fibril growth phase, where the initial phase can be rapidly accelerated if a seed is added [49-51]. Although the polymerization process is not fully understood, some intermediate Aβ species such as Aβ-derived diffusible ligands (ADDLs) and protofibrils (which are thought to be the immediate precursors of fibrils) have been isolated [52]. The ADDLs are globular oligomers that range in size from trimers to 24-mers [53]. Protofibrils can be visualized by EM as short fibril intermediates with a diameter of 6-8 nm and a length less than 150 nm [54]. Several lines of evidence have shown that these intermediate species are more toxic than the amyloid fibrils [43]. Despite that only two amino acid residues differ between Aβ40 and Aβ42, they show significantly different aggregation properties. Aβ42 is much more prone to aggregate and forms fibrils at lower concentrations than Aβ40 [55]. This might be an explanation for the observation that Aβ42 is the initial species deposited in AD and DS brains [21, 22]. Studies

on Aβ polymerization have revealed regions in Aβ of particular importance for fibril formation [56]. Amino acid residues 16-20 in Aβ, corresponding to the KLVFF sequence, are essential for Aβ fibrillization, and removal of these residues perturbed fibril formation [57]. Residues in the N terminus of Aβ (1-9) are less important for fibril formation [58].

Despite the substantial information existing about Aβ fibril formation *in vitro*, many questions remain regarding how and where Aβ polymerization starts *in vivo*. The concentration of Aβ in CSF and plasma are much lower (0.1-1 nM) than the concentration required for *in vitro* polymerization [59]. The probability that Aβ will aggregate into insoluble species in the brain may depend on several different factors, such as high local concentrations of Aβ in certain brain areas, pH, and metal ions concentrations [60]. Moreover, Aβ-binding proteins might play a role and facilitate Aβ aggregation.

Plaque-Associated Proteins

A range of different proteins have by immunohistochemical methods been shown to co-localize with SPs. Among the most well characterized, regarding their effect on Aβ polymerization as well as binding-sites to Aβ, are apoE, α1-antichymotrypsin (ACT) and complement component C1q. Extracellular matrix (ECM) components such as heparan sulfate proteoglycans (HSPGs), laminin and collagens are also commonly found in SPs. The majority of the identified plaque-associated proteins have only been shown to be co-deposited with Aβ by immunohistochemical means, and no further evidence exist as to whether these proteins are directly associated with the Aβ fibrils.

Plaque-associated proteins could promote Aβ fibril formation by seeding the polymerization process or stabilize intermediates and fibrils. Alternatively, they could interfere with the aggregation by inhibiting fibril growth. Proteins such as apoE and ACT have been suggested to act as "pathological chaperones", factors that promote the deposition of Aβ into SPs [61]. Several studies have shown that both apoE and ACT are integral parts of SPs [62-64] and that they catalyze Aβ fibril formation *in vitro* [65-67]. *In vivo* studies of transgenic mice overexpressing mutant APP crossed with mice lacking endogenous apoE show substantially decreased Aβ deposition [68, 69]. Further, studies in transgenic mice carrying a FAD mutation in the *APP* gene (PDAPP mice) have shown that ACT facilitate amyloid deposition and promote cognitive impairment [70-72].

Laminin, the major component of the basement membrane, is known to accumulate in the SPs [73] and have in several studies been shown to inhibit Aβ fibril formation [74-76] and possibly also induce disaggregation of preformed fibrils [77, 78]. Other ECM components such as the HSPGs have been found to associate with amyloid deposits in other tissues in addition to SPs [79, 80] and many of them such as perlecan and agrin, enhanced the rate of Aβ fibril formation [81, 82]. A number of collagens and proteins carrying collagen domains have been found to associate with Aβ deposits such as; collagen XVIII [83], which was recently found to carry heparan sulfate side-chains [84], collagen type IV [85], shown *in vitro* to inhibit Aβ fibril formation [86]. Also, the proteins acetylcholinesterase (AChE) [87] and

C1q [88] containing collagen domains, have been shown to activate the classical complement pathway *in vitro* and thereby induce inflammatory processes [89].

Plaque-associated proteins may also interact with the clearance of SPs by protecting Aβ fibrils from degradation. Serum amyloid P (SAP) component, which is found in all types of amyloid including SPs [90], is highly protease resistant and has been suggested to protect amyloid from degradation [91]. HSPGs maintain fibril stability by protecting fibrillary Aβ from proteolysis *in vitro* [92]. The α2-macroglobulin protein has been implicated biochemically in binding to Aβ and hypothesized to affect the degradation/clearance of SPs [93].

To summarize, some of these proteins have been identified as plaque-associated proteins, and shown with both *in vitro* and *in vivo* models to affect Aβ polymerization as well as its deposition into amyloid deposits. Despite this, it is difficult to draw any conclusions about their actual impact on the disease progression. Today one drug that affects Aβ polymerization is under trial, the glycosaminoglycans (GAG) mimetic Alzhemed™. This compound mimics sulphated GAGs and prevents amyloid build up by competing with natural occurring HSPGs, that otherwise bind and promotes amyloid formation and/or prevents clearance by increased protease resistance. This thesis is focused on a novel plaque-associated protein, termed CLAC (collagenous Alzheimer amyloid plaque component) [94], and its role in the pathogenesis of AD. In contrast to many of the other plaque-associated proteins, CLAC has been suggested to bind only Aβ deposits suggesting a more specific role in the pathogenesis of AD. CLAC is a member of the collagen superfamily, which will be reviewed in the following sections.

COLLAGEN BIOLOGY

The Collagen Superfamily

The collagen superfamily represents a diverse group of proteins which are located in the ECM and makes up about one-third (by weight) of all proteins in the mammalian tissue [95, 96]. They play important roles in maintaining the structure of various tissues and create an ordered ECM structure by associating with themselves and other matrix components. In addition to their supportive role, the collagens are involved in cellular functions such as adhesion, migration and differentiation. At least 27 different collagen types have been described, and they are classified together because they share several biochemical and histological features [97, 98]. They all have a unique structural element, consisting of three polypeptide chains that are winded around a common axis to form a right-handed super helix, the so-called triple helix [99]. The polypeptide chains have a repeating Gly-X-Y amino acid sequence, in which X and Y are often proline and 4-hydroxyproline, respectively. In addition to the triple-helical domains, all collagens contain non-collagenous domains. Examples of these are von Willebrand factor and thrombospondin domains.

Most collagens form supramolecular assemblies such as fibrils and networks, and can be divided into several subfamilies based on these suprastructures: fibril-forming, network-forming, fibril-associated, beaded filament-forming, anchoring fibrils and transmembrane collagens. The transmembrane collagens are an emerging group of collagens that will be discussed in more detail below. There are some inconsistencies in the nomenclature of collagens and it has been proposed that all proteins containing collagenous sequences should be considered as members of the collagen family regardless of their function. Still there are a number of proteins, the collagen-like proteins, which possess collagenous sequences but are not defined as collagens since they are not part of the ECM [100]. This group of proteins includes the complement component C1q [101], AChE [102], ficolins [103], macrophage scavenger receptors type I and II [104, 105], collectins [106] and ectodysplasin A [107].

Structure and Assembly of the Triple Helix

The collagen triple helix is a linear structure composed of three left-handed polyproline-II type helices that are staggered by one residue with respect to each other [99, 108]. A glycine residue at every third position in the Gly-X-Y sequence is a prerequisite for the tight packing of the helix, since glycin is located in the center of the triple helix. The side chains of residues in the X and Y position are located at the surface of the molecule and are the major determinants for recognition and binding to other proteins [109].

Before assembly into the triple helix, the unfolded polypeptide chains undergo extensive posttranslational modifications in the endoplasmatic reticulum. Prolines and lysines are enzymatically hydroxylated by prolyl 4-hydroxylase and lysyl hydroxylase, respectively [110, 111]. Crosslinking of the hydroxylysines by lysyl oxidase between the different chains contributes to the stability as well as rendering the collagen highly insoluble. Moreover, the

hydroxylysines are often glycosylated. The thermal stability of collagens are dependent on the imino acids, proline and 4-hydroxyproline, and the stability of a particular collagen type increases as the content of these residues increases [112, 113]. 4-hydroxyproline contributes more than proline to the stability due to its formation of intramolecular hydrogen bonds. Moreover, high hydroxyproline content can compensate for destabilizing factors such as interruption in the triple helix structure by non-collagenous sequences. A number of hereditary connective tissue diseases such as osteogenesis imperfectia [114, 115], which is characterized by bone fragility, have been associated with mutations in collagens. The most common mutations are point mutations that result in substitution of a glycine residue in the repeating Gly-X-Y sequence by another amino acid, which causes destabilization and/or defective folding of the triple helix [116].

The *in vivo* assembly of the collagen triple helix is a complicated process and occurs in a zipper-like manner [117]. Successful formation of the triple helix requires that only *trans*-peptide bonds are present in all prolyl residues. This conversion from *cis* to *trans* is catalyzed by cis-trans prolyl isomerase [118], which catalyzes the isomerization of imide bonds in proteins. The reaction is energy demanding and is the rate limiting step in the process forming the triple helix [119]. A nucleus for the assembly of the triple helix is usually formed in the C-terminal region and propagation occurs towards the N terminus. Recently it was shown that the N terminus in collagen XIII could induce folding of the entire molecule from the N-terminal end [120], and similar observations have been seen for other collagens [121, 122]. After triple helix formation the molecules are secreted and assembled into supramolecular structures such as fibrils, filaments and networks that form the supporting matrix of different tissues. The triple helix is resistant to different proteases [123], and can therefore only be degraded by specific collagenases. *In vitro*, purified collagens spontaneously self-assemble at neutral pH and at room temperature in a strongly concentration dependent-manner and forms fibrils that are identical to those seen *in vivo*.

The Transmembrane Collagen Family

The first integral membrane proteins found to contain collagenous sequences were the type I and II macrophage scavenger receptors. Currently the transmembrane collagen family includes 8 members: the closely related collagen XIII [124], XVII [125], XXIII [126] and XXV [94] (Figure 3), and the more distantly related ectodysplasin A [107], the class A macrophage scavenger receptors [104, 105], the MARCO receptor [127] and the newly discovered colmedins (collagen repeat plus olfactomedin domain containing proteins) [128]. They are all predicted to be type II transmembrane proteins, consisting of a short N-terminal cytosolic domain (except collagen XVII), a single transmembrane domain and a large ectodomain mainly composed of collagenous (COL) domains separated and flanked by non-collagenous (NC) domains. The ectodomain is released from the plasma membrane by proteolytic cleavage carried out by a group of cell surface enzymes collectively referred to as sheddases, including the furin convertase family [129]. The collagen types XIII, XVII, XXIII and XXV are all known to release their ectodomains but it is not known whether they all are processed by the same sheddase. Many of them contain a furin consensus sequence, and furin

has been suggested to be the responsible protease [94, 120, 130, 131] except for collagen XVII where cleavage is performed by the TACE (tumor necrosis factor-α convertase) protease family [132].

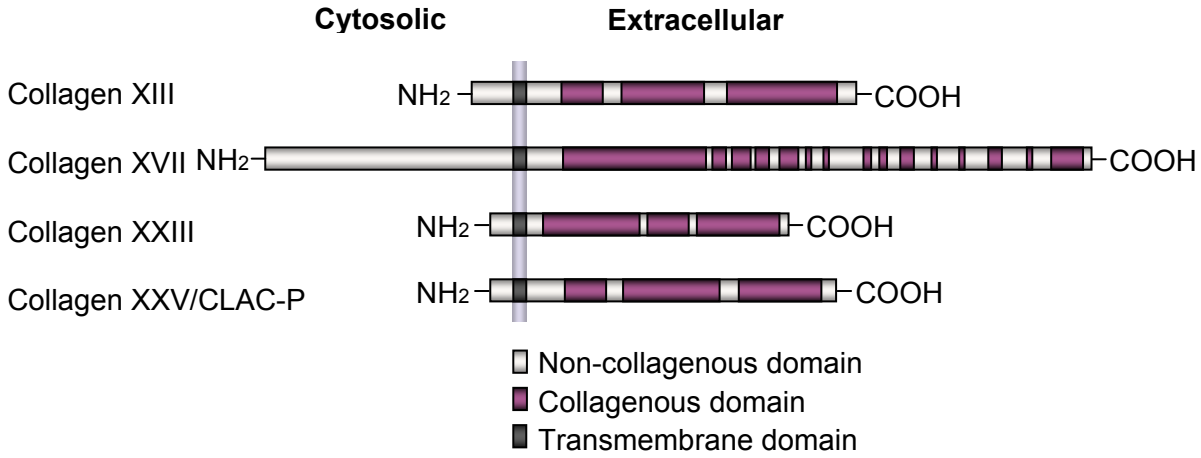

Figure 4: Schematic presentation of the molecular structures of collagenous transmembrane proteins.

The triple helix of all collagenous transmembrane proteins is composed of three identical polypeptide chains and, in contrast to other collagens, the trimerization is believed to proceed in the N- to C-terminal direction [120, 122]. Conserved sequences next to the membrane-spanning region (in the NC1 domain), are important, but not crucial, for trimerization since additional trimerization triggering domains have been found in more C-terminal regions for collagen types XIII, XXIII and XXV [133, 134].

The function of most of these newly discovered collagens is largely unknown. They are unique due to their dual existence as a membrane-bound and a soluble form. Hence, they might interact with their environment in multiple ways, both as cell surface receptors and/or as soluble ligands and matrix component. Collagen type XIII and XVII show a wide tissue expression and have been suggested to be involved in cellular adhesion or act as receptors for soluble ligands. The ectodomain of collagen type XIII has been found to interact with the integrin-type collagen receptor α1β1 [135]. The physiological function of collagen XXV/CLAC-P is not known but it appears to be involved in the pathology of AD which will be discussed in the following sections.

THE CLAC PROTEIN

Background

In 1997, a protein, referred to as AMY, was described in the Alzheimer's disease brain [136]. It was recognized by monoclonal antibodies (mAbs) raised against AD brain extracts enriched in paired helical filaments (PHFs). Instead of labeling NFTs and hyperphosphorylated tau, these antibodies recognized plaque-like lesions in AD brain sections and a 100 kDa protein species on western blots [136]. At first, AMY was thought to be part of a new plaque-like lesion specific for AD, since no co-localization with Aβ containing plaques was observed [136]. However, it was later shown that AMY co-localized with the majority of Aβ positive plaques in AD brain [137]. Although some immunohistochemical data existed on AMY [138, 139], the molecular identity of this 100 kDa protein was unknown. In 2002, a novel Alzheimer amyloid plaque protein, CLAC (collagenous Alzheimer amyloid plaque component), was described [94]. It was also discovered by screening mAbs raised against insoluble fractions prepared from AD brain. A striking similarity existed between AMY and CLAC regarding molecular size, immunostaining patterns and biochemical features. In 2003, we showed that AMY is identical to the CLAC protein (**paper I**). Hereafter, AMY will be referred to as CLAC.

Features of CLAC

CLAC is the soluble ectodomain derived from the precursor protein CLAC-P, also termed collagen type XXV. CLAC-P is a type II transmembrane protein belonging to the growing transmembrane collagen family [140]. Through proteolytic cleavage mediated by furin, the C-terminal ectodomain CLAC is released and deposited with the SPs in AD brain. CLAC-P shows high structural similarities with collagen XIII and XXIII (Figure 3), with a putative transmembrane domain, 3 collagenous domains (COL1-COL3) and 4 non-collagenous domains (NC1-NC4) (Figure 4). The biological function of CLAC-P is unknown and so far expression has been confined to neurons [94]. However, expression profile analysis of expressed sequence tags (ESTs), using the ProfileViewer at the NCBI database (www.ncbi.nlm.nih.gov), suggests that CLAC-P is expressed in other tissues. This is further supported by results obtained by multiple tissue expression array analysis (Dillner unpublished data). One remarkable feature of collagen XIII is the extensive alternative splicing generating several different transcripts [141]. This is probably an attribute also of CLAC-P since it contains as many as 41 exons. Several alternatively transcripts of the CLAC-P gene (*COL25A1*) have been reported [94] (Dillner unpublished data) and as predicted by the evidenceViwer at the NCBI database. Currently, the longest isoform of CLAC-P is composed of 654 amino acids. When expressed recombinantly in mammalian cell lines, CLAC migrates with a slightly larger molecular weight than expected, about 75 kDa, and multimers corresponding to dimers and trimers are seen under non-reducing conditions (**paper II**) [94, 142].

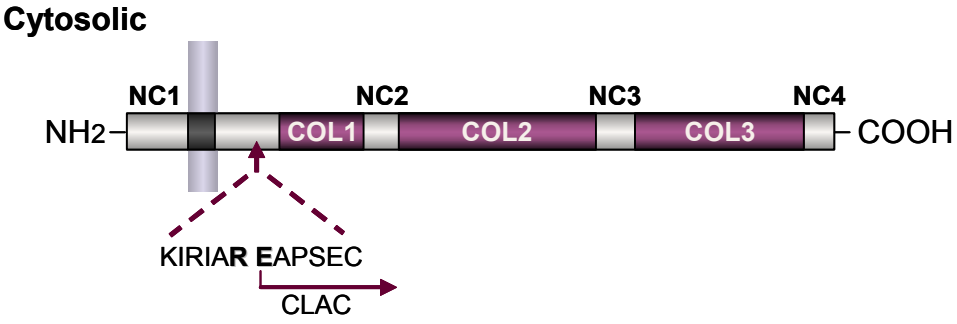

Figure 4: Schematic presentation of CLAC-P/collagen XXV. CLAC is released by furin cleavage at the KIRIAR sequence in the NC1 domain, starting with a glutamate residue.

CLAC in the Pathogenesis of Alzheimer's Disease

In human AD brain CLAC co-purifies with Aβ and other plaque components in sodium dodecyl sulphated (SDS) insoluble fractions isolated from human AD brains [94] (**paper I**). It migrates predominantly as a 50 and a 100 kDa species, probably representing monomers and dimers, respectively [94] (**paper I**). It does not co-purify with similar fractions prepared from brains lacking AD pathology, or in brains with other neurodegenerative disorders, indicating the specificity of CLAC to AD. In brain sections, CLAC shows co-localization with SPs and is not found associated with NFTs. Neither is it found in amyloid deposits in cerebral vessels or other structures of the brain [94, 136, 137]. CLAC is present in a subset of plaques, mainly in the mature and neuritic plaques commonly present in severe AD cases. Here, CLAC immunoreactivity is usually observed surrounding the plaques whereas the core portion is usually devoid of immunoreactivity. CLAC is not present in the diffuse plaques, mainly composed of amorphous, non-fibrillar, Aβ42 [94, 137]. A recent study performed on AD and DS brains, suggests that CLAC co-localize preferentially more with an "early" type of plaques, composed of loosely dispersed Aβ42 fibrils, distinct both from diffuse and mature plaques [143]. In the same study, CLAC was found to be associated with plaques in the brains of transgenic mice overexpressing FAD associated variants of *APP* and *PS1* genes (PSAPP mice) [144]. Immunohistochemical studies on brain sections from FAD cases carrying mutations in the *PS1*, *PS2* or *APP* gene were performed before the identity of AMY was known. No differences between FAD and SAD cases regarding AMY positive plaques could be observed [139].

Studies performed *in vitro* using a solid-phase binding immunoassay and recombinant CLAC shows that CLAC binds to aggregated Aβ, but not to soluble Aβ [94]. The interaction is blocked in the presence of sodium chloride, implying that the interaction is partly ionic [94] (**paper II**). Positively charged residues, both in the NC2 (**paper II**) and in the COL1 domain [142], in CLAC play a major role in the binding to Aβ. Today's literature does not tell us anything about sequences in the Aβ peptide crucial for binding to CLAC. Although some *in vitro* data regarding the CLAC/Aβ interaction exists, the role of CLAC in the disease progression is rather unexplored. A recent *in vitro* study investigating Aβ polymerization in

the presence of CLAC suggests that CLAC might have an anti-amyloidogenic role in the pathogenesis of AD, since less Aβ fibrils were observed in the presence of CLAC [142]. This was further supported by the finding that CLAC binds preferentially to an "earlier" type of plaques, mainly composed of Aβ42, and as suggested by Kowa *et al*. CLAC might inhibit further maturation of these into mature plaques [143]. Based on data presented in this thesis (**paper III and IV**), a novel function of CLAC in the pathogenesis of AD will be discussed.

AIMS OF THE STUDY

Deposition of Aβ into SPs is a critical event in the pathogenesis of AD and also one of the hallmarks of the disease. Although a large knowledge exists about the Aβ fibril formation *in vitro* a lot remains to be elucidated regarding formation of SPs in the brain. A number of non-Aβ components have been found to co-deposit with SPs but their role in the disease progress is not determined. The overall aim of this thesis was to characterize the novel plaque-associated protein CLAC, and obtain a better understanding of its role in the pathogenesis of AD.

The specific aims of the studies were:

Paper I: To determine the molecular nature of AMY, a protein found in SPs in AD brain.

Paper II: To biochemically characterize CLAC, described in paper I, and identify sequences in CLAC involved in binding to Aβ.

Paper III: To search for binding motifs in the Aβ peptide important for the interaction with CLAC, and to investigate the effect of CLAC on the Aβ fibril elongation process.

Paper IV: To study the effect of CLAC on Aβ fibrils in order to better understand the role of CLAC in the pathogenesis of AD.

RESULTS AND DISCUSSION

Identification of AMY as CLAC and Biochemical Characterization of CLAC

In 1997 mAbs generated against insoluble NFTs isolated from AD brain were found to label plaque-like lesions in AD brain [136]. The immunoreactivity was later shown to co-localize with the majority of SPs in AD and DS brains but the identity of the antigen was unknown [137]. Therefore we considered it of interest to identify the protein, termed AMY, responsible for this immunoreactivity.

In **paper I** a protocol for the enrichment of insoluble Aβ [145] was used with some modifications for isolation of AMY. AMY reactivity co-purified with Aβ and was exclusively found in the SDS-insoluble fraction in AD brains, whereas AMY reactivity was completely absent in non-AD individuals. Separation of the SDS-insoluble fraction by HPLC followed by amino acid sequence analysis of AMY-containing fractions did not result in unambiguous identification. Some other proteins were identified such us Aβ, ferritin, creatine kinase and sequences corresponding to collagens. To circumvent the difficulties in purifying AMY, an alternative approach was used based on biochemical and immunohistochemical similarities with the newly discovered plaque-associated protein CLAC-P/collagen XXV [94]. To address whether the AMY antibody also recognized CLAC, a gene encoding a splice variant containing 580-residue of the precursor protein of CLAC, CLAC-P, was cloned from a human brain cDNA library and expressed in a mammalian cell line. The AMY antibody recognized the expressed CLAC, and AMY and CLAC mAbs labeled identical protein species on immunoblots of SDS-insoluble AD brain extracts. Moreover, similar structures were stained by the two antibodies on consecutive AD brain sections. These results made us draw the conclusion that AMY was identical to CLAC.

To obtain more thorough information regarding the nature of CLAC, an expression and purification system for production of recombinant CLAC, containing a C-terminal myc/His tag, was devised (**paper II**). Human embryonic kidney (HEK) 293 cells were chosen, since they express sufficient levels of the enzyme prolyl 4-hydroxylase which is necessary for biosynthesis of collagens [146]. The purity of CLAC isolated from cell culture media, was estimated to be ~95% by Coomassie blue staining. Generation of the soluble ectodomain of CLAC from CLAC-P has previously been suggested to be mediated by a furin convertase [94]. This was verified by N-terminal sequencing of purified CLAC, where the starting amino acid obtained was Glu113. This residue is proceeded by the furin recognition sequence $_{107}$KIRIAR$_{112}$ [147] and thus, the cleavage occurs between Arg112 and Glu113. CLAC-P and CLAC migrated as 85 kDa and 75 kDa protein species, respectively, on SDS-PAGE (**paper I** and **II**). These molecular weights are higher than expected from calculation of the primary sequences alone, but retarded migration is a common feature of collagens containing a high amount of imino residues [148]. The retarded migration might also be due to the presence of carbohydrate groups since we show that CLAC was sialylated (**paper II**). Glycosylation is a common feature of collagens and other ECM components.

CLAC isolated from human brain migrates predominantly as 50 and 100 kDa protein species (**Paper I**) [94], distinct from recombinantly expressed CLAC. It is possible that the species isolated from brain represents truncated CLAC variants since several isoforms of CLAC have been identified [94] (Dillner unpublished data). This is supported by the finding that the CLAC homologue collagen XIII undergoes complex splicing and generates several isoforms [141]. Another possible modification is crosslinking of lysines by reducing sugars. This is a common feature of long lived proteins, and products from this non-enzymatic reaction, advanced glycation endproducts (AGE) [149] are present in SPs. Alternatively, CLAC in brain might be partially degraded. In **paper I**, the epitope of the AMY155 mAb was partially determined and found to be located within the COL2 domain between residues 311-358. The molecule is not extensively truncated in the N terminus since COL2 of CLAC is present in the SPs. Thus, CLAC isolated from brain might contain several modifications and should therefore not be directly compared with recombinantly expressed CLAC.

To investigate whether CLAC expressed in HEK293 cells adopts the triple-helical structure characteristic for collagens, a range of different biochemical methods were used (**paper II**). Amino acid analysis was performed on purified CLAC, and 11% and 49% of total proline and lysine residues were found to be hydroxylated. Hydroxylation is necessary for the stability and formation of the triple helix and the extent of hydroxylation vary depending on collagen type and expression system used, i.e. mammalian or insect cells. Due to the triple-helical structure, collagens are partly protease resistant [123, 150], and this feature was also investigated for CLAC. CLAC was digested by pepsin and analyzed by SDS-PAGE. The size of the observed fragments agreed with the expected COL domain fragments, and the cleavage sites were further verified by N-terminal sequencing. The collagen character of CLAC was confirmed by circular dichroism (CD) spectroscopy analysis showing a typical collagen spectrum with a large minimum at 198 nm and a minor peak at 225 nm [151]. Under non-reducing conditions, purified recombinant CLAC exits as SDS-stable dimers and trimers, indicating that CLAC harbors a triple-helical structure. Under reducing conditions CLAC was predominantly found as monomers suggesting that disulfide-bonds are important for holding the polypeptide chains together. The 100 kDa CLAC species found in brain is unaffected by reducing agents, SDS and FA, and we suggest that covalent modifications are responsible for the increased stability.

Interaction of CLAC with Aggregated Aβ

In **paper II** we showed that CLAC produced in HEK293 cells adopts a collagen conformation. Correct folding of CLAC is crucial since much of the work presented in this thesis aimed at studying the interaction between CLAC and Aβ. A solid-phase binding immunoassay (**paper II**) and surface plasmon resonance (SPR) spectroscopy (**paper III**) were used for analysis of CLAC/Aβ interaction.

In 2002 Hashimoto *et al.* reported that CLAC binds to aggregated Aβ, but not to monomeric Aβ [94]. We confirmed these findings by optimizing a similar solid-phase binding immunoassay, but instead of using culture media containing CLAC we used purified CLAC (**paper II**). CLAC bound to aggregated Aβ1-40 in a concentration-dependent manner and the interaction was blocked by increasing concentrations of sodium chloride (completely abolished with 0.5 M NaCl). It has been speculated that CLAC has a higher affinity for Aβ1-42 aggregates [143] but we showed that CLAC binds equally well to both Aβ1-40 and Aβ1-42.

In **paper III** we thoroughly investigated the CLAC/Aβ interaction by quantifying the binding using SPR spectroscopy. The principle behind SPR spectroscopy is outlined in figure 10 in *methodological considerations*, and a typical sensorgram is illustrated in figure 5. Biotinylated monomeric Aβ1-40 or Aβ1-42 as well as aggregated Aβ1-42 were immobilized to streptavidin sensor chips. CLAC bound to aggregated Aβ in a concentration-dependent manner, while less interaction was seen with monomeric species. Regarding the kinetics of CLAC binding to Aβ aggregates, neither the simple model nor the rearrangement model available in the BIAcore evaluation software represented the data well. Possible reasons for the difficulties calculating an exact K_d value could be due to re-binding of CLAC to the chip, slow dissociation and heterogeneity of the chip surface. An apparent K_d value was calculated to range from 0.7 to 5.0 nM using a Langmuir binding model. K_d values in this range suggest a strong affinity of CLAC to Aβ aggregates.

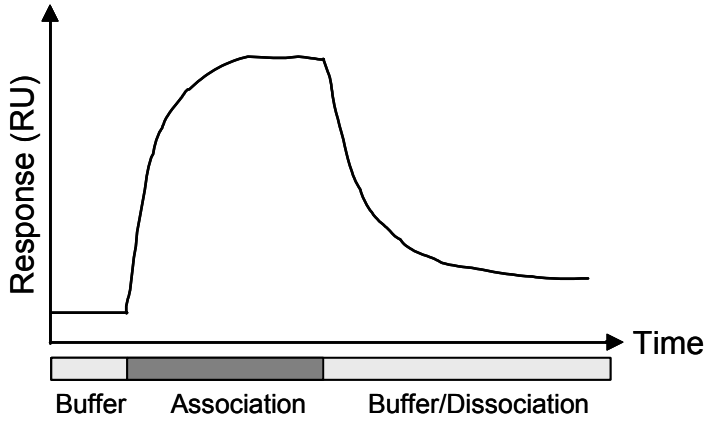

Figure 5: A typical sensorgram where the analyte is injected at a constant concentration during the association phase. During this injection the signal is correlated to binding of analyte to ligand. After injection bound analyte dissociates in buffer flow. Adapted from Karlsson et al. *J. Mol. Recognit.* 2004.

Binding Motifs in CLAC and Aβ

In **paper II** we found an eight amino acid long sequence, $_{181}$LIKRRLIK$_{188}$, in NC domain 2 of CLAC to be crucial for binding to Aβ. The motif was found when short and overlapping peptides corresponding to the NC2-4 domains were synthesized on a membrane and screened for binding to iodinated Aβ. The principle of the method is illustrated by the cartoon if figure 9 in the section of *methodological considerations* [152]. The NC2-4 domains were chosen since they are more specific for CLAC than the COL domains that are found also in non-plaque associated collagens. Sequences of the NC1 domain were not investigated, since this part contains the transmembrane domain and is not a part of the shedded ectodomain.

The involvement of the LIKRRLIK sequence in binding to Aβ was studied by generating CLAC mutants were the LIKRRLIK sequence was deleted or exchanged for other amino acids (Figure 6). The mutants lacking the proposed binding site, ΔLIKRRLIK-CLAC, did not bind to aggregated Aβ in the solid-phase binding immunoassay. These results were verified by a sedimentation assay, where Aβ fibrils in solution were incubated with wild type (Wt) CLAC or ΔLIKRRLIK-CLAC followed by centrifugation. In comparison with Wt-CLAC, only ~30% of ΔLIKRRLIK-CLAC co-sedimented with Aβ fibrils. CLAC binding to Aβ fibrils could be competed in a dose-dependent manner by a 12-mer peptide, QQLIKRRLIKGD, containing the Aβ-binding motif.

The importance of the LIKRRLIK sequence was further analyzed by replacing it with the VIKRRTFQ sequence, a sequence found in NC2 in the homologous collagen XIII. An intermediate binding to Aβ fibrils was observed in this case. The LIKRRLIK sequence contains 4 positively charged residues (underlined) compared to VIKRRTFQ that contains 3. These findings suggest that positively charged residues at this position in CLAC are crucial for Aβ binding. Collagen XIII has not been found associated with SPs despite the high overall sequence similarity with CLAC and the sequence similarities in NC2. Whether this is due to a lower expression of collagen XIII in the brain or absence of additional Aβ binding motif(s) possibly present in CLAC is not known. Possibly, collagen XIII might lack binding motif(s) for other SP components facilitating the interaction between CLAC and Aβ.

Recently, another Aβ-binding motif, $_{127}$KRGKRGRR$_{134}$, in COL1 was proposed by Osada *et al*. [142]. This motif resembles the LIKRRLIK sequence by containing a cluster of positively charged amino acids. We generated a mutant lacking this motif and analyzed it with respect to Aβ binding (**paper II**). In contrast to the study by Osada *et al*., our mutant did not show impaired Aβ binding. One possibility for this discrepancy could be that different splice variants of Wt-CLAC were used in the two studies. A CLAC variant containing all exons was used by Osada *et al*., and a C-terminally truncated splice form lacking 52 amino acids was used here. Further, the mutants were generated in two different ways; deletion of the whole motif (**paper II**) versus exchanges for amino acids by proline residues [142]. Hence, the proposed binding-sites in COL1 and NC2 domains might be differently exposed in two types of Wt-CLAC, thereby reflecting the differences in binding to Aβ.

A similar CLAC species, containing the missing 52 amino acid residues, have been cloned by us and compared with the splice variant used in **paper II**. No differences in binding affinity for Aβ between the two were observed (Söderberg unpublished data), suggesting that the Aβ-binding motif is not affected by the missing 52 amino acid residues. This does not reveal whether there are any structural differences between the two CLAC species and it would therefore be of interest to delete the two proposed binding-sites in the respective CLAC species. In summary, both proposed Aβ-binding motifs contain clusters of positively charged amino acids and strengthen the hypothesis that the CLAC/Aβ interaction partly depends on ionic interactions. An ionic interaction between C1q and Aβ has been demonstrated [153]. C1q, like CLAC, contains a cluster of positively charged amino acid residues, $_{14}$AGRPGRRGRPGLK$_{26}$, within the N-terminal collagenous domain. This sequence has been suggested to bind to the negatively charged residues in the N terminus of Aβ [154] (see Aβ sequence Figure 2).

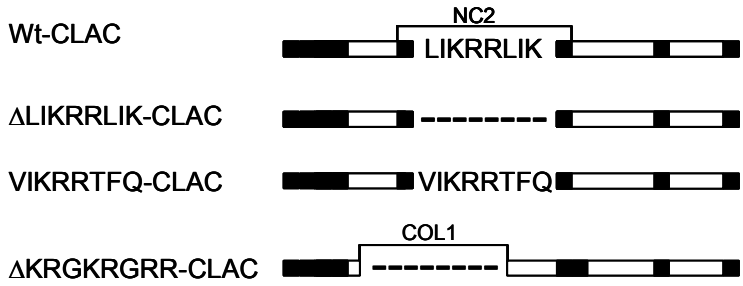

Figure 6: Outline of the different CLAC mutants used in **paper II**. The NC domains are shown as black boxes. Deletions of amino acids are represented by Δ.

In **paper III** we wanted to extend the CLAC/Aβ interaction study by searching for sequences in Aβ involved in CLAC binding. We used SPR spectroscopy and a solid-phase binding immunoassay and several different Aβ fragments were studied (Figure 7). Results obtained by SPR spectroscopy showed that CLAC bound to the different Aβ peptides in a concentration-dependent manner with strong binding to Aβ1-42, intermediate binding to Aβ1-40, Aβ1-28, and Aβ12-28, and considerably less binding to the N-terminal Aβ1-16 fragment and the C-terminal Aβ29-40 fragment. A similar binding pattern was observed in the solid-phase binding immunoassay. The main difference was that Aβ1-40 and Aβ1-28 bound CLAC with similar affinity as Aβ1-42. The amount of aggregated peptides immobilized to the plate was estimated by ThT fluorescence. If the amount of immobilized aggregated peptide was taken into account, Aβ12-28 and Aβ10-20 could be considered strong CLAC binders.

Further, the effects of mutations in the shortest fragment required for CLAC binding, Aβ10-20, was studied. The mutants analyzed were Aβ10-20$^{AA\ 13,14}$ and Aβ10-20$^{AA\ 19,20}$, with alanines substituted for the His13 and 14, and the Phe19 and 20, respectively. Neither CLAC binding nor fibril formation ability was affected by the His to Ala substitutions. In sharp contrast, both CLAC binding and ThT fluorescence were reduced for the Aβ10-20 Phe to Ala

mutant. The two Phe residues 19 and 20 have previously been identified as crucial for fibril formation [57, 155, 156] and also verified here.

Osada *et al*. show the implication of the N-terminal part of Aβ in CLAC/Aβ interaction [142]. This is in contrast with data presented in **paper III** that show no binding to Aβ1-16. Aβ1-16 did not form fibrils under the conditions used in our study and we do not exclude the possibility that CLAC could bind to an aggregated form of Aβ1-16. Thus, binding of CLAC to Aβ is intimately correlated to the aggregation state of Aβ. The importance of aggregates is also reflected by the ability of CLAC to bind other amyloidogenic peptides, such as the non-Aβ component of α-synuclein (NAC) and amylin as shown in **paper II**. In **paper II** we also show by two different methods that CLAC has only intermediate affinity to amylin aggregates. We speculate that the lower binding obtained with amylin fibrils are due to the low number of negatively charged amino acids in amylin. Hence, CLAC's ability to bind to non-Aβ fibrils and the binding dependence on aggregation state suggests that CLAC primarily recognize structure of the fibrils rather than on the amino acid sequence. We also hypothesize that binding of CLAC to fibrils are dependent on negatively charged residues.

Figure 7: Primary sequence of Aβ1-42. Bars cover the residues included in the fragments used in **paper III**. Substituted residues are presented in bold and negatively charged residues are underlined.

The Role of CLAC in the Pathogenesis of AD

The biological function of CLAC and its role in AD remains largely unknown. CLAC is found in mature plaques and not in the earliest Aβ deposition, the diffuse plaques lacking β-sheet structure [136, 137]. This indicates that CLAC comes in at later stages of the disease progression. However, a recent study by Kowa *et al*. showed preferentially co-localization of CLAC to deposits mainly composed of loosely packed Aβ42 fibrils. Based on these observations they suggest that CLAC might be a relatively early component of SPs [143]. It is difficult to obtain a mechanism for CLAC's role in the pathogenesis of AD solely from immunohistochemistry and therefore we studied the effect of CLAC on Aβ fibrils and the fibril formation process (**paper III** and **IV**).

Previous reports have demonstrated that SPR spectroscopy is a useful method for analysis of Aβ fibril elongation [157]. The advantage of SPR spectroscopy over other methods is that binding is studied in real time and analysis is not dependent on any fibril binding dyes such as ThT. In **paper III** a procedure to study Aβ fibril elongation in the presence of CLAC was devised. In the absence of CLAC, the rate of Aβ fibril elongation was concentration-dependent while treatment of Aβ fibrils with 50 nM and 100 nM CLAC decreased the rate of Aβ fibril elongation by 20% and 30%, respectively. This is in keeping with previous ThT fluorescence data suggesting that CLAC inhibits fibril formation in the elongation phase [142]. A model for how this occurs could be that CLAC binds to the ends of the emerging fibrils and thereby decreases the rate of fibril elongation.

In **paper IV** we wanted to address the effect of CLAC on Aβ fibrils. Incubation of preformed fibrils with CLAC led to an increased turbidity, indicating the formation of larger aggregates. This finding was supported by EM analysis where a higher number of larger aggregates were observed in the presence of CLAC. Moreover, when Aβ fibrils were sedimented by centrifugation in the presence of CLAC, more Aβ was found in the pellet compared to control samples where no CLAC was added. To investigate whether the effect of CLAC was specific for Aβ fibrils, fibrils formed from NAC, were incubated with CLAC. Turbidity measurements showed that CLAC had a similar effect on NAC fibrils (Dahlqvist unpublished data), indicating that CLAC may affect other amyloid-forming peptides in a similar way. Hence, these data suggested that CLAC assembles Aβ fibrils into larger aggregates, a function that has not previously been described for other plaque-associated proteins. A number of compounds have been reported to disaggregate Aβ fibrils [78, 158]. These experiments have been based on ThT fluorescence analysis, where the reduction in fluorescence has been attributed to a disaggregation of Aβ fibrils. When CLAC was added to preformed Aβ fibrils and analyzed by ThT, a reduction in ThT fluorescence was observed already within one hour. This reduction was not accompanied by a decrease in the amount of Aβ fibrils as determined by SDS-PAGE/Coomassie staining. Hence, the reduced ThT fluorescence seen in the presence of CLAC is not due to a decrease amount of Aβ fibrils. Rather, ThT and CLAC compete for the same binding-sites on Aβ fibril and the high concentration of ThT in the fibril bundle could lead to quenching. We also investigated the effect of CLAC on freshly solubilized Aβ using the ThT fluorescence assay. CLAC did not affect the lag-phase of Aβ

polymerization, in agreement that CLAC does not bind to monomeric Aβ. A reduction in ThT fluorescence was observed at the end-point (5 days) and probably reflects binding of CLAC to fibrils as seen when CLAC is mixed with preformed fibrils. Interestingly, in the paper by Osada *et al*., a reduction in ThT fluorescence in the presence of CLAC was observed before Aβ fibrils formed despite several studies have shown that CLAC does not bind to monomeric Aβ. The potential problems with ThT will be discussed further in the section of *methodological considerations*.

To investigate the biological relevance of CLAC's assembly of preformed fibrils, a protease resistance assay was used to address whether the assembly resulted in protection of Aβ from proteolysis (**paper IV**). When Aβ fibrils were treated with proteinase K in the absence of CLAC only a low amount of Aβ remained. In contrast, a high amount of Aβ remained after overnight digestion in the presence of CLAC. Thus, the assembled CLAC-containing aggregates are resistant to proteolytic degradation. CLAC did not have a protective effect on soluble Aβ, further showing the preferential binding of CLAC to aggregated Aβ. We suggests that CLAC may act in a similar way *in vivo* and play a role in the accumulation and persistence of the plaques by inhibiting proteolytic degradation from *in vivo* enzymes such as neprilysin, a known Aβ-degrading enzyme [159]. In support of this contention is the increased protease resistance observed in CLAC-positive plaques [160].

GENERAL DISCUSSION & FUTURE PERSPECTIVES

The pathology and genetics of AD, as well as studies in transgenic mice and cell lines suggest that Aβ, the main component of the SPs, plays a key role in the development of AD. Despite the wealth of information resulting from studies concerned with Aβ aggregation *in vitro* and *in vivo*, the mechanism whereby Aβ monomers assembles into oligomers, larger aggregates and finally into SPs remains unclear. Several studies have focused on proteins co-deposited with SPs and their involvement in the formation and persistence of SPs. Some of these co-deposited proteins have been shown to directly modulate Aβ aggregation either by promoting or inhibiting Aβ's polymerization. The most well-studied proteins with respect to Aβ aggregation are apoE and ACT. For these two proteins both *in vitro* and *in vivo* data suggest a direct interaction with Aβ.

This thesis is focused on the novel plaque-associated protein CLAC, which is a member of the transmembrane collagen family. CLAC is an extracellular protein shedded from the cell surface from its precursor protein CLAC-P, and has been found to co-deposit with SPs in AD brain. CLAC has not, as expected from its extracellular localization, been found in association with intracellular aggregates, such as Lewy bodies (composed principally of α-synuclein protein). Despite *in vitro* data demonstrating that CLAC has an intermediate affinity for amylin (**paper II**), CLAC is only found in brain associated with extracellular Aβ amyloid deposits. Absence of CLAC in amyloid deposits in other tissues may be due to low expression levels of CLAC in these tissues and/or an altered stability of CLAC in the periphery. It is also possible that SPs contain yet unknown compounds that interact with CLAC and facilitate its deposition onto SPs. The regional specificity of CLAC deposition is further demonstrated by the lack of CLAC-immunoreactivity in cerebrovascular amyloid.

Our interest in studies on CLAC started when we found that CLAC was the antigen causing the AMY-immunoreactivity found in AD and DS individuals [136]. To determine the biochemical properties of CLAC an expression system was devised, and secreted CLAC was purified from cell culture media. Purified CLAC was used for the initial characterization, where CLAC displayed typical collagen features (**paper I**). The physiological function of CLAC is not known and limited data exists on CLAC and its role in AD. We therefore asked the following questions: (i) is there an Aβ-binding motif in CLAC (ii) is there a corresponding CLAC-binding motif in Aβ and, (iii) what, if any, are the effects of CLAC binding to Aβ. An Aβ-binding sequence, LIKRRLIK, was found within the non-collagenous domain 2 of CLAC (**paper II**). We also show that CLAC binds to aggregated, but not monomeric Aβ. Further, binding of CLAC to Aβ aggregates was shown to decrease the rate of Aβ fibril elongation (**paper III**). We hypothesize that CLAC in this case binds to the ends of the growing fibrils and inhibit further incorporation of monomeric Aβ, thereby decreasing the overall rate of elongation. *In vivo*, this could have an impact on the SPs by preventing further SP maturation and growth. An additional effect of CLAC binding to Aβ may be that CLAC assembles Aβ fibrils into bundles by binding to their surfaces and link them together into larger aggregates (**paper IV**). These large CLAC-containing Aβ aggregates were shown to be more resistant to

proteases. The same mechanism may be valid also *in vivo*, as supported by findings by Kowa *et al*. [160]. The increased protease resistance of Aβ cold be due to shielding of the fibrils by CLAC molecules. CLAC-induced packing of Aβ fibrils can also make them inaccessible to the Aβ-degrading enzymes or clearance by microglia. Based on the results presented in this thesis a possible mechanism of action for CLAC in AD is illustrated in figure 8.

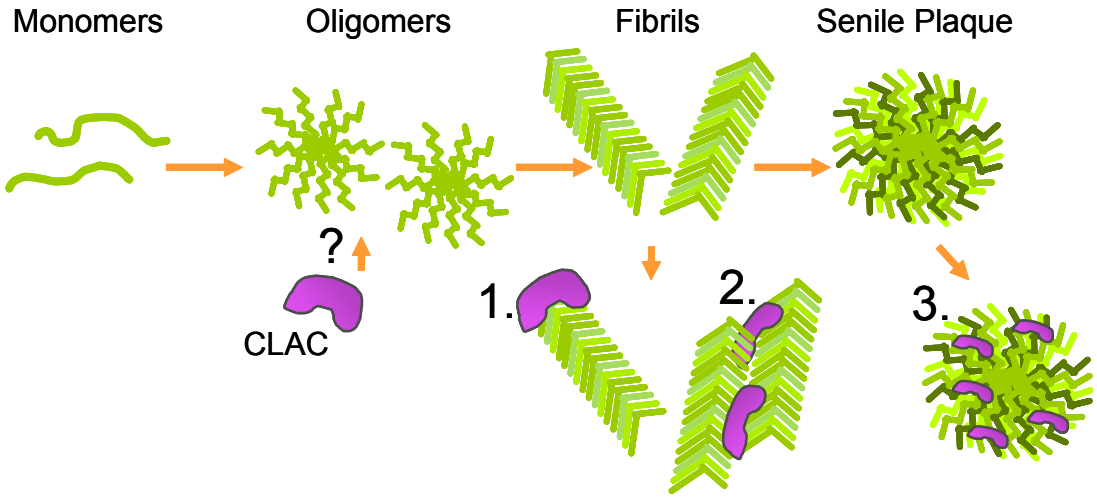

Figure 8: A suggested model of CLAC's role in the pathogenesis of AD, based on the findings in this thesis. No binding of CLAC is seen to Aβ monomers, which will polymerize into oligomers and further into fibrils. Whether CLAC binds to oligomers or protofibrils is not known. We show that CLAC binds to Aβ fibrils and CLAC's role may be the following: **1.** CLAC may bind to the end of the growing Aβ fibril and inhibit further elongation (**paper III**), and/or **2.** binds to Aβ fibrils and assemble them to larger aggregates (**paper IV**). **3.** Binding of CLAC may therefore have an effect on the persistence of SPs by making them more protease resistant (supported by *in vitro* data **paper IV**).

We are beginning to understand the role of CLAC in AD but still many questions remain. Is the deposition of CLAC in AD brain caused by increased expression of CLAC due to missense mutations in the CLAC gene or caused by an increased release of soluble CLAC from CLAC-P? Does CLAC bind to intermediates formed in the Aβ polymerization process, such as soluble Aβ oligomers and protofibrils? What is the physiological function of CLAC? Transgenic mice with an altered CLAC-expression will provide answers to some of these questions. Meanwhile, model *in vivo* systems such as primary neuronal cell cultures could provide information about the effects of CLAC on Aβ-induced toxicity. The therapeutic potential of CLAC in AD is dependent on whether blocking of the CLAC/Aβ interaction is desired. Peptidomimetics based on the LIKRRLIK sequence may be useful for interfering with the CLAC/Aβ interaction, since a 12-mer peptide containing this motif is able to compete with CLAC for Aβ binding (**paper II**). It is possible that disruption of the plaques will lead to release of soluble Aβ species that are more toxic to neurons than the actual deposits. CLAC-binding to SPs might preserve Aβ in its fibrillar form and thereby lower the amount of toxic species, thus having a protective effect.

In conclusion, CLAC is a collagen that binds to aggregated forms of Aβ resulting in the formation of larger protease resistant aggregates. One explanation to the accumulation and resistance to proteases of SPs in AD brain may be that CLAC acts in a similar way *in vivo*.

METHODOLOGICAL CONSIDERATIONS

Several methods have been used in this thesis and detailed descriptions of the procedures are found in the respective papers. This is a summary of some of the methods, focusing on the less conventional techniques, their advantages and limitations.

Production of recombinant CLAC in HEK293 cells

Collagens undergo extensive post-translational modifications; i.e. glycosylation, crosslinking and hydroxylation. Hydroxylation of proline and lysine residues, carried out by prolyl 4-hydroxylase, is a requirement for the stability and formation of the collagen triple-helical structure. The commonly used protein expression systems, such as bacteria and yeast, lack prolyl 4-hydroxylase and Sf9 insect cells express insufficient levels of it. To circumvent the low expression levels in insect cells, collagens can simultaneously be co-expressed with human prolyl 4-hydroxylase, and thereby provide a good system for production of recombinant collagens. We used a mammalian expression system, human embryonic kidney (HEK) cells, for the expression of CLAC. HEK293 cells have adequate levels of prolyl 4-hydroxylase and offer a good system for the production of collagens [146]. A stable HEK293 cell line expressing CLAC-P fused to a C-terminal myc/His tag were generated. Secreted CLAC was purified from culture media by a two-step purification scheme. In order to obtain CLAC with a high purity, the second purification step was based on affinity for the 6xHis-tag. The advantage of using tagged CLAC is that it increases the ease of purification and that levels of CLAC mutants can conveniently be adjusted using the myc antibody. However, the tag itself may influence the structure of the protein, resulting in altered characteristics of the tagged protein vis-à-vis the non-tagged molecule.

SPOT synthesis method

The SPOT method permits rapid and parallel synthesis of a large numbers of peptides on a cellulose support and the subsequent screening of binding interactions [152]. Common applications are epitope mapping as well as investigations on interactions between proteins/peptides and nucleic acids. Peptides are synthesized on a cellulose membrane carrying free hydroxyl groups that are covalently bound to the carboxylic group of an alanine that will act as a spacer. One cycle includes the following steps: coupling of a desired amino acid to the free amino terminus of the previous amino acid; activation of the carboxylic group of the added amino acid in order to form an amide bond; derivatization with a protection group of the added amino acid in order to prevent reaction with the activated carboxylic group; and removal of the protection group to generate a free amino terminus for the following amino acid to be coupled.

In this thesis, the SPOT technique was used for screening of an Aβ-binding motif in CLAC. An array of 10 residues long peptides with a 8 residues overlap, covering sequences of the NC2-NC4 domains of CLAC, was synthesized on a nitrocellulose membrane according to the SPOT manual (Sigma-Genosys). Bolton Hunter reagent was used for the iodination of the Aβ1-40 and 1-42 peptides. Screening was performed by incubation of the SPOT membrane

with iodinated Aβ peptides followed by autoradiography. Importantly, the method is limited to the synthesis of linear peptide sequences. Hence, epitopes or motifs with discontinuous sequences will omit detection using this method and is better assayed by SPR spectroscopy. The general strategy of the SPOT technique is shown in figure 9.

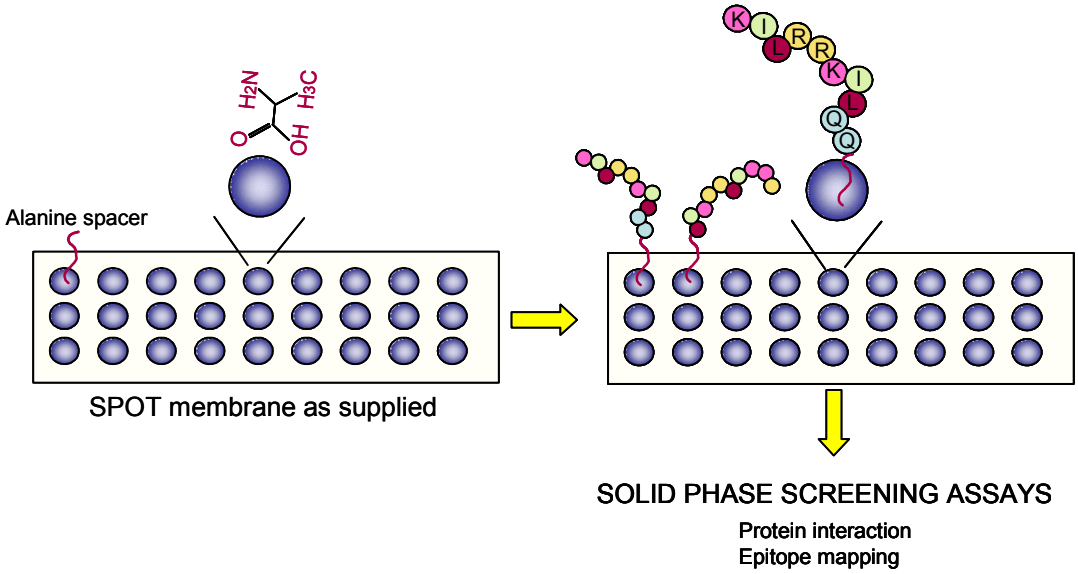

Figure 9: Cartoon illustrating the principle of SPOT-synthesis. The SPOT membrane is supplied with an alanine spacer anchored to the membrane to which the peptides can be synthesized in the C- to N-terminal direction.

Solid-phase binding immunoassay

Solid-phase binding methods are commonly used for studying protein-protein interactions. When using this method, only a qualitative estimate of the affinity is obtained. To calculate binding parameters such as dissociation constants (K_d values), SPR spectroscopy is better suited. In the solid-phase binding assay, the protein is coated onto a microtiter plate and incubated with a solution containing the protein under investigation. Binding is detected by antibodies to the applied protein followed by colorimetric detection. In this thesis we have analyzed the interaction of CLAC to different fibril forming peptides (Aβ, amylin and NAC). Several coating techniques and plates have been used and for detailed descriptions we refer to the respective papers. In most studies, the peptides were allowed to aggregate before coating onto microtiter wells and drying at 37°C overnight. The linear range obtained for binding of CLAC to Aβ aggregates was narrow, ranging from 1-20 nM.

Quantification of Aβ fibrils

Congo red (CR) binding

Amyloid is composed of cross β-sheet structure that possess specific staining properties. The most common way to visualize amyloid in tissue is by staining with the histological dye CR that binds to amyloid because of the extensive β-sheet structure. CR bound to fibrils exhibit red and green birefringence under polarized light. CR can also label fibrils in solution

followed by measurements of absorbance, since CR-binding to fibrils induces a spectral change. Turbidity, thioflavin T binding and electron microscopy are other commonly used methods for analyzing fibrils.

Turbidity

An easy way to measure the presence of aggregates in a solution is to measure the turbidity by absorbance at 355 nm. In contrast to many other methods such as CR or ThT, using this method all types of aggregates are measured and no ordered β-sheet structure is required. We analyzed the effect of CLAC on preformed Aβ aggregates using turbidity measurements (paper IV). The preformed Aβ fibrils used were separated from soluble Aβ by centrifugation and the total amount of aggregated Aβ were not expected to increase upon incubation with CLAC. An increase in turbidity would arise from the formation of larger aggregates.

Thioflavin T (ThT) assay

The fluorophore ThT binds to amyloid and can be used for studies on the Aβ fibril formation process. Binding of ThT to fibrils produces a shift in its emission spectrum where a fluorescent signal is proportional to the amount of amyloid formed [161]. ThT is also commonly used for studies aimed at investigating different compounds effect on the Aβ polymerization process. A number of compounds have been suggested to either inhibit Aβ polymerization and fibril elongation or induce disaggregation of already formed fibrils, based on a reduced ThT fluorescence signal [78, 158, 162].

We have used the ThT assay to examine the effect of CLAC on Aβ fibrils and the Aβ polymerization process (paper IV). Twenty µl of preformed Aβ1-40 fibrils (100 µM) were incubated in the presence or absence of 100 nM of CLAC and after different time-points a 10 µM ThT solution (10 mM phosphate buffer, 150 mM NaCl, pH 6.0) was added. Fluorescence spectra of ThT were acquired with excitation at 440 nm and emission at 490 nm on a fluorescence spectrometer. To examine the effect of CLAC on Aβ polymerization, a solution mainly composed of monomeric Aβ1-40 in 100% DMSO was diluted in TBS to a final concentration of 25 µM followed by addition of CLAC (final concentration 100 nM). The mixture was allowed to shake (600 rpm) at room temperature for 5 days and at different time-points aliquots of 50 µl were analyzed for Aβ fibril formation by ThT fluorescence.

The reduction in ThT fluorescence observed when preformed Aβ fibrils were incubated in the presence of CLAC was not accompanied by a lower amount of Aβ fibrils as measured by turbidity, SDS-PAGE and electron microscopy. We do not believe that the reduced ThT values are caused by disaggregation of the Aβ fibrils into smaller aggregates (monomers and oligomers). Instead we suggest that the reduced fluorescence is caused by (i) either a competition of ThT and CLAC for a common binding-site or (ii) quenching of fluorescence due to an altered ThT binding, as has been suggested for the Aβ-binding compound norihydroguaiaretic acid (NDGA) [163]. NDGA has been shown to be an effective inhibitor of Aβ fibril elongation and induce disaggregation of Aβ fibrils [158]. Similar effects have been observed with laminin [78]. When laminin was tested in parallel with CLAC in our

studies, a similar reduction in ThT fluorescence was demonstrated and no reduction in the amount of Aβ fibrils was seen (Dahlqvist unpublished observations). In agreement with Osada *et al*., a reduced ThT signal was obtained when CLAC was incubated with monomeric Aβ peptide followed by the polymerization process (paper IV) [142]. We speculate that this reduction is partly based on similar interactions between CLAC and ThT. In summary, ThT is a suitable method for investigation of Aβ polymerization by itself but when used together with other compounds it should be carefully evaluated and be used in combination with other methods.

Surface plasmon resonance (SPR) spectroscopy

SPR spectroscopy is used for studies on protein-protein interactions, such as ligand-receptor or antigen-antibody interactions [164]. It is also a useful method for analysis of Aβ fibril elongation [165]. In SPR spectroscopy, the ligand is immobilized onto the sensor chip surface, and the analyte flows through a narrow channel in contact with the surface. A change in the refractive index of the solution occurs due to the increase in mass when the analyte binds to the immobilized ligand. The SPR detector monitors the change in refractive index in real time and the changes are recorded and presented in a graph called "sensorgram". The principle of the method is outlined in figure 10. SPR spectroscopy offers a range of advantages, the binding reaction is monitored in real time, high sensitivity makes it possible to detect the binding of small molecular weight peptides and importantly, no labeling is required. A pure analyte sample is important since additional compounds may interact with the immobilized ligand. Non-specific binding and surface heterogeneity are some of the limitations of SPR spectroscopy. SPR spectroscopy analysis was used for detailed studies on the CLAC/Aβ interactions, such as CLAC's affinity to different aggregation states of the Aβ peptide. In addition we studied binding to different fragments of Aβ and the rate of Aβ fibril elongation in the presence or absence of CLAC (paper III).

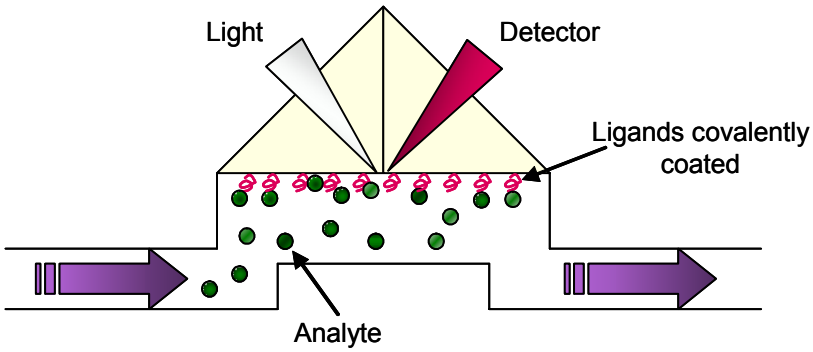

Figure 10: Schematic of SPR biosensor assay. The ligand is immobilized onto sensor chip surface, and the analyte flows through in contact with the surface.

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