Characterization of the Alzheimer’s Disease-associated CLAC Protein and Identification of an Amyloid β-Peptide-binding Site*

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amyloid β-peptide (Aβ) deposition into amyloid plaques is one of the invariant neuropathological features of Alzheimer’s disease. Other proteins co-deposit with Aβ in plaques, and one recently identified amyloid-associated protein is the collagen-like Alzheimer amyloid plaque component CLAC. It is not known how CLAC deposition affects Aβ plaque genesis and the progress of the disease. Here, we studied the in vitro properties of CLAC purified from a mammalian expression system. 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. Purified CLAC was also used to investigate the interaction between CLAC and Aβ. Using a solid-phase binding assay, we show that CLAC bound with a similar affinity to aggregates formed by Aβ1–40 and Aβ1–42 and that the interaction was impaired by increasing salt concentrations. An 8-residue-long sequence located in non-collagenous domain 2 of CLAC was found to be crucial for the interaction with Aβ. These findings may be useful for future therapeutic interventions aimed at finding compounds that modulate the binding of CLAC to Aβ deposits.

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized by selective neuronal loss associated with intracellular neurofibrillary tangle formation and extracellular amyloid plaques (1). The major constituents of the amyloid plaques are fibrils formed from the 40–42-residue amyloid β-peptide (Aβ) (2). Aβ is generated from the type I transmembrane Alzheimer amyloid precursor protein by two consecutive proteolytic cleavages. The aspartyl protease β-secretase BACE generates the N terminus of Aβ, whereas the C terminus results from the proteolytic action of the γ-secretase complex (3). Data from genetic and biochemical studies suggest that accumulation of the longer and more amyloidogenic species of Aβ, Aβ42, is a primary event in the development of AD (4). For instance, autosomal dominant forms of early-onset familial AD appear to result from an increased production of Aβ42 (5), and total levels of brain Aβ42 correlate with cognitive decline in AD (6).

Several proteins other than Aβ have been shown by immunohistochemical methods to be associated with AD amyloid plaques (7). Some of these plaque-associated proteins, e.g. apolipoprotein D (8, 9), apolipoprotein E (10), and α1-antichymotrypsin (11), modulate peptide aggregation. In addition, constituents of the extracellular brain matrix, e.g. the heparan sulfate proteoglycans (12–14), laminin (15, 16), and collagen type IV (17), accumulate in amyloid plaques and bind to Aβ. The appearance of some of these plaque-associated proteins may be indicative of a local inflammatory response to the amyloid, and others may promote Aβ aggregation or stabilize the amyloid plaques. Recently, a novel plaque-associated protein, CLAC (collagen-like Alzheimer amyloid plaque component), was identified using antibodies raised against insoluble amyloid fractions purified from AD brain (18). In mammalian cells, CLAC is generated from the precursor protein CLAC-P/collagen type XXV by a furin-like cleavage event. Secreted CLAC has been shown to bind aggregated Aβ1–42 in an in vitro binding assay (18). We have recently reported that the plaque-associated protein AMY is identical to the CLAC protein (19). The AMY/CLAC protein is co-localized with Aβ in plaques both in AD and Down’s syndrome brains (20, 21), and no immunoreactivity is found in cerebral vessels and brain tissue from patients with other neurodegenerative or neuropsychiatric disorders.

The collagens contain one or more characteristic triple-helical domains consisting of the tripeptide repeat Gly-X-Y, with X and Y being frequently proline and hydroxyproline residues, respectively (22). These residues are required for the correct conformation of the specific triple-helical structure, and underhydroxylation has been shown to lead to instability of the helix (23). CLAC-P belongs to a collagenous transmembrane protein subgroup that currently has eight members (24): collagen types XIII, XVII, XXIII, and XXV; macrophage scavenger receptor types I and II; a scavenger receptor with C-type lectin; and ectodysplasin A. These collagens have a short cytosolic N terminus, a transmembrane domain, and one or more extracellular collagenous domains interspersed by non-collagenous domains. CLAC-P resembles collagen type XIII in its overall arrangement by having a short intracellular domain and an extracellular ectodomain with three collagenous domains (COL1–3) and four short non-collagenous domains (NC1–4). The two collagens share 43% sequence identity at the amino acid level. Collagen type XIII has a wide tissue distribution (25), whereas CLAC-P has neuron-specific expression (18). In this study, we expressed and purified CLAC to study its biochemical characteristics and interaction with Aβ. We show that CLAC expressed in human embryonic kidney (HEK) 293...
cells is glycosylated, forms a triple-helical structure, binds to heparin, and binds with the same efficiency to Aβ-(1–40) and Aβ-(1–42) aggregates. Finally, we identify an Aβ-binding motif in CLAC, the LIKRRLIK sequence located within the NC2 domain.

**EXPERIMENTAL PROCEDURES**

**Construction of CLAC-P and Mutant Expression Vectors—**A splice variant of CLAC-P/follagen type XXV consisting of 580 residues was cloned from a human brain cDNA library into the pcDNA3/mycHis expression vector, which contains a C-terminal myc/His tag, as described previously (19). Mutant CLAC expression constructs were generated by site-directed mutagenesis using PCR according to the manufacturer’s protocol (Stratagene, La Jolla, CA). Three CLAC mutants were made. One truncated CLAC variant (ALIKRRLIK-CLAC) has a deletion of residues 181–188, which correspond to the LIKRRLIK sequence (GenBank®/EBI accession number AF293914), and one substituted variant (VIKRRFTQ-CLAC) has the LIKRRLIK sequence replaced with VIKRRFTQ. The VIKRRFTQ sequence in the NC2 domain of collagen type XIII is located in an identical position (amino acids 253–260 in GenBank®/EBI accession number NP_005194) to the LIKRRLIK sequence in the NC2 domain of CLAC. The third mutant generated (DKRR-CLAC) is identical with TBS-β (5000 M NaCl were dialyzed against buffer containing TBS and 1% bovine serum albumin (BSA; Sigma) for 2 h, followed by three washes with TBS containing 0.05% Tween 20 (TBS-T; Sigma). Purified CLAC was diluted in blocking buffer to concentrations of 5, 10, and 20 nm. Samples (100 µl) were applied to heparin-coated and control wells and allowed to bind for 2 h at room temperature. The wells were washed (3×) and incubated with a 1:50,000 dilution of sheep anti-mouse antibody (Amersham Biosciences) overnight in blocking buffer for 1 h. The procedure was repeated using horseradish peroxidase-conjugated anti-mouse secondary antibody (1:5000), followed by three rinses with TBS-T and detection of antibody binding with 100 µl of peroxidase substrate solution (3,3',5,5'-tetramethylbenzidine, Pierce). The reaction was stopped with 100 µl of 2 M H₂SO₄, and color development was measured in a FLUOstar Galaxy microplate reader at 450 nm.

**Pepsin Digestion and N-terminal Sequencing—**Purified recombinant CLAC was subjected to pepsin digestion. Cleavage was performed overnight at 37 °C with 1 µg/ml pepsin (Sigma) at a substrate/enzyme ratio of 10:1 in 10 mM HCl. The cleavage mixture was neutralized with 100 mM Tris-HCl (pH 10.5) before analysis by SDS-PAGE. Samples were visualized by Coomassie Blue staining and subjected to N-terminal sequencing on an Applied Biosystems 491 Proteomic System (Applied Biosystems, Foster City, CA). The purity of CLAC was analyzed by SDS-PAGE and immunoblotting using anti-Myc antibody. Heparin binding was further confirmed by a solid-phase assay using 5 µg of heparin (50 µl of a heparin stock of 0.1 mg/ml; Sigma) allowed to bind overnight to a 96-well plate (MaxiSorp, Nunc, Naper-ville, IL) at 4 °C. Wells were blocked (including blank wells without heparin) by incubation with 300 µl of blocking buffer containing TBS and 1% bovine serum albumin (BSA; Sigma) for 2 h, followed by three washes with TBS containing 0.05% Tween 20 (TBS-T; Sigma). Purified CLAC was diluted in blocking buffer to concentrations of 5, 10, and 20 nm. Samples (100 µl) were applied to heparin-coated and control wells and allowed to bind for 2 h at room temperature. The wells were washed (3×) and incubated with a 1:50,000 dilution of sheep anti-mouse antibody (Amersham Biosciences) overnight in blocking buffer for 1 h. The procedure was repeated using horseradish peroxidase-conjugated anti-mouse secondary antibody (1:5000), followed by three rinses with TBS-T and detection of antibody binding with 100 µl of peroxidase substrate solution (3,3',5,5'-tetramethylbenzidine, Pierce). The reaction was stopped with 100 µl of 2 M H₂SO₄, and color development was measured in a FLUOstar Galaxy microplate reader at 450 nm.

**CD Spectroscopy—**Samples were dialyzed against buffer containing 10 mM NaF and 10 mM Tris-HCl (pH 7.4). Samples at a concentration 1 µM were used. CD spectra between 260 and 180 nm were recorded at 25 °C in 1-cm pathlength quartz cells using a Jasco J-815 spectropolarimeter.

**Amino Acid Analysis—**Purified recombinant CLAC was hydrolyzed with 6 M HCl for 24 h at 110 °C. Hydrolyzed samples were analyzed on an amino acid analyzer (Biochrom 20 Plus) using ninhydrin chemistry. Solid-phase Binding Assay—Human peptides Aβ-(1–40), Aβ-(1–42), amylin-(1–37), and NAC-(1–35) (gen-α component) (Bachem AG, Bubendorf, Switzerland) were added to 100 µl PBS to a concentration of 0.1 mg/ml (Aβ-(1–42), amylin, and NAC) or 0.2 mg/ml (Aβ-(1–40)), and incubated overnight at 37 °C with shaking (500 rpm). A thioflavin T-based assay was used to confirm that the samples had aggregates (26, 27). Five µg of the respective aggregated peptides (50 µl of a peptide solution of 0.1 mg/ml) was transferred to a 96-well plate (MaxiSorp) and dried overnight at 37 °C. The wells were incubated with 300 µl of blocking buffer (pH 7.4) for 2 h at room temperature and washed three times with TBS-T. CLAC was diluted in blocking buffer to concentrations of 10, 5, and 2.5 µM. Protein concentrations of the CLAC samples were measured by the micro BCA assay (Pierce) and incubated for 2 h at room temperature. CLAC binding was quantified according to the method used in the solid-phase heparin binding assay described above. Competition experiments were performed with the 12-mer peptide QQLIKRRLIKGD, which includes the proposed Aβ-binding site located in the NC2 domain of CLAC. Five µM CLAC was incubated with 200 µl of the following peptide concentrations: (1.0, 10.0, and 25 µM) and incubated in wells coated with aggregated Aβ-(1–42) for 2 h, followed by detection as described above.

**CLAC Precipitation Assay—**The Aβ-(1–40) and amylin-(1–37) peptides were diluted in a 100% Me₃SO stock solution to 50 µM in PBS and incubated at 37 °C with shaking (500 rpm) overnight. Aggregation was confirmed with the thioflavin T fluorescence assay (26, 27). The aggregated peptides were diluted in PBS to a final peptide concentration of 25

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Expression and purification of the CLAC ectodomain. A, shown is a schematic outline of the purification scheme used to purify CLAC from HEK 293 cells stably expressing CLAC-P-Myc/His. Conditioned medium was concentrated on a cation-exchange column (HiTrap SP/FF), and Myc/His-tagged CLAC was purified on a nickel-chelating column. B, shown are the results from SDS-PAGE analysis of purified CLAC separated on a 4–12% BisTris gel and stained with Coomassie Blue. C, purified CLAC was subjected to Edman degradation, and the sequence obtained confirmed the expected cleavage by a furin-like protease.

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μM. One-hundred μl of the solution was mixed with 200 μl of culture medium from HEK 293 cells stably expressing CLAC-P-Myc/His and incubated for 2 h at room temperature with shaking (300 rpm). As a negative control, CLAC culture medium was incubated with PBS. Aggregates and coprecipitating CLAC were pelleted by centrifugation at 5000 × g for 10 min. Pellets were dissolved in Laemmli sample buffer (Sigma) and separated on a 4–12% BisTris gel. CLAC coprecipitating with the peptide aggregates was quantified by enhanced chemiluminescence detection using a CCD camera. A similar procedure as described above was applied for coprecipitating CLAC and ΔIKRRLIK-CLAC with Aβ(1–40). Aβ(1–40) was prepared as described and diluted to 25 μM. Aβ aggregates were divided into tubes (50 μl), mixed with purified CLAC and ΔIKRRLIK-CLAC (100 μl), and diluted to a final concentration of 2 nM in 1% BSA in phosphate buffer. Samples were incubated at 37 °C for 2 h with shaking (300 rpm). Centrifugation and analysis using anti-Myc antibody were performed as described above. Protein concentrations of samples were determined by amino acid analysis.

SPOT’s Membrane Analysis—An array of peptides 10 residues long with overlaps of 2 residues was synthesized on a nitrocellulose membrane according to the SPOTs manual (Sigma) (28). The peptides synthesized covered the sequences of the NC2–4 domains of CLAC-P. Approximately 20 μg of Aβ(1–40) or Aβ(1–42) was iodinated using [125I] Bolton-Hunter reagent (Amersham Biosciences AB). The iodination reaction was carried out in 0.1 M borate buffer (pH 8.5) for 15 min at 0 °C. The aggregation state was assayed with thioflavin T, showing that aggregates were present in the solution. The reaction was quenched by the addition of 1 M ethanolamine, and the reaction mixture was separated on a PD10 column (Amersham Biosciences AB) equilibrated with TBS. Before use, the membrane was incubated in 100% methanol, washed three times with TBS, and blocked in TBS-T for 1 h. The SPOTs membrane was incubated with iodinated Aβ peptides in blocking buffer overnight at room temperature (final peptide concentration of ~0.1 μg/ml). The membrane was washed with changes of TBS-T. An Eastman Kodak dual emulsion film (together with a Biomax intensifying screen) was exposed to the filter overnight at ~70 °C. Quantification of the membrane was performed by densitometry.

RESULTS

Purification of Recombinant CLAC—To characterize CLAC and to study its interaction with Aβ, we developed a method for the expression and purification of recombinant CLAC. The 580-residue splice variant of CLAC-P used herein has previously been cloned by us (19) from a human brain cDNA library and further cloned into a mammalian expression vector carrying a C-terminal myc/His tag. The purification method is schematically summarized in Fig. 1A. Briefly, CLAC fused to a C-terminal myc/His tag was purified from conditioned medium from HEK 293 cells stably expressing CLAC-P-Myc/His using a cation-exchange column and a nickel-chelating column. Purification on the nickel column was performed in the presence of 2 M urea throughout all steps. When urea was excluded from the buffers, the affinity of CLAC for the nickel column was markedly decreased, as indicated by the elution of CLAC at low concentrations of imidazole. The samples were dialyzed and subjected to SDS-PAGE analysis. Staining with Coomassie Blue (Fig. 1B) and silver staining (data not shown) showed that CLAC migrated with an apparent molecular mass of ~75 kDa. The purity was estimated to be ~95%. Secretion of the CLAC ectodomain from the membrane-bound precursor protein has been suggested to be mediated by a furin-like protease (18). N-terminal sequencing was used to obtain the exact cleavage site of CLAC when expressed in HEK 293 cells. The sequence obtained verified that the cleavage, as expected for a furin protease, occurred between Arg112 and Glu113, after the KIRIAEAPSE sequence (Fig. 1C).

Amino acid analysis showed that 11% of the total prolyl residues and 49% of the total lysyl residues were hydroxylated (Table I). By comparison, in collagen type XIII, ~72% of the total prolyl residues and 30% of the total lysyl residues are hydroxylated (29). These differences are probably attributed to the expression system: the HEK 293 cells used here and the Sf9 cells for the collagen type XIII studies. Overall, the amino acid compositions determined correlated well with the expected

### Table I: Amino acid composition of purified recombinant CLAC

| Amino acid | Residues/ectodomain | Theoretical composition |
|------------|---------------------|-------------------------|
| Ala        | 18.5                | 18                      |
| Arg        | 19.7                | 19                      |
| Asn (Asp)  | 31.6                | 30                      |
| Cys ND     | 4                   |                         |
| Glu (Gln)  | 67.0                | 62                      |
| Gly        | 143.2               | 147                     |
| His        | 10.3                | 11                      |
| Ile        | 18.0                | 17                      |
| Leu        | 25.7                | 25                      |
| Lys        | 18.2                | 38                      |
| Lys-OH     | 17.3 (49%)          |                         |
| Met        | 11.3                | 13                      |
| Phe        | 4.6                 | 3                       |
| Pro        | 74.8                | 95                      |
| Pro-OH     | 9.3 (11%)           |                         |
| Ser        | 13.1                | 17                      |
| Thr        | 11.5                | 12                      |
| Trp        | 0.0                 | 1                       |
| Tyr        | 2.5                 | 1                       |
| Val        | 5.1                 | 5                       |

*ND, not determined
The presence of carbohydrate groups was detected using horseradish peroxidase-conjugated streptavidin, followed by ECL visualization. B, a modification of the protocol used in A detected sialic acid groups on CLAC (>). C, purified CLAC was immunoblotted using different digoxigenin-labeled lectins. Note the strong positive signal obtained from the MAA lectin. The control lane contains CLAC, to which only the anti-digoxigenin antibody was added. PNA, peanut agglutinin; G. nivalis, a modification of the protocol used in A detecting sialic acid groups on CLAC (>). No significant background staining was observed. The band with a molecular mass of 180 kDa obtained upon incubation with MAA is probably a result of MAA binding to a dimeric form of CLAC (see below). The heparin-binding consensus sequence consists of the BBXB motif, where B represents a basic residue, and X represents any residue (31). By analyzing the CLAC sequence, we identified two heparin-binding consensus sequences in the COL1 domain of CLAC. Other clusters of basic residues also exist within the NC2 domain. To study the binding of CLAC to heparin, conditioned medium from cells stably expressing CLAC-P was applied to a heparin column. CLAC bound with an intermediate affinity to the heparin column, eluting at a concentration of 0.3–0.5 M NaCl in PBS (Fig. 3A). The binding of CLAC to heparin was further confirmed by incubating purified CLAC with immobilized heparin in a solid-phase binding assay. CLAC bound to heparin in a concentration-dependent manner (Fig. 3B), and no binding of CLAC to immobilized BSA was observed (data not shown).

Pepsin Resistance of CLAC—The collagens are partly resistant to proteases, a feature commonly used for their isolation and purification. Here, we used pepsin to determine the resistance of CLAC to proteases. A schematic presentation of CLAC-P and its predicted pepsin-resistant fragments are outlined in Fig. 4A. Cleavage with pepsin was carried out overnight at 37 °C, and resistant fragments with molecular masses of ∼45, 33, 21, 13, and 5 kDa were visualized after separation by SDS-PAGE and Coomassie Blue staining (Fig. 4B). The bands with molecular masses of ∼21, 13, and 5 kDa correspond to the expected pepsin-resistant fragments as outlined in Fig. 4A. However, the most intense bands obtained after pepsin digestion were seen migrating at 45 and 33 kDa. These bands were subjected to N-terminal sequencing. The sequence obtained from the 45-kDa fragment corresponds to the C-terminal residues of the NC2 domain, suggesting that the 45-kDa fragment represents the COL2, NC3, COL3, and NC4 domains of CLAC (Fig. 4, A and B). The N-terminal sequence obtained from the 33-kDa band corresponds to the last residues in the NC3 domain (Fig. 4, A and B); and thus, this fragment might represent a stable dimer composed of the COL3 and NC4 domains.

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Multimer Formation and Structure of CLAC—To study whether CLAC forms multimers, purified CLAC was subjected to SDS-PAGE under reducing and nonreducing conditions and in the presence or absence of SDS, followed by Western blot analysis using anti-Myc antibody. Immunoreactive bands were observed at 180 and 240 kDa, probably representing dimers and trimers of CLAC, respectively (Fig. 5A). These bands were not observed in the presence of reducing agents (Fig. 5A). Interestingly, a dimer is often seen when CLAC is purified from human AD brain (18, 19). The formation of dimers and trimers might be a result of disulfide-bond formation involving the NC1 domain, with 2 cysteine residues, and the NC4 domain, with 1 cysteine residue. The addition of SDS had no effect on oligomer formation (Fig. 5A). To further investigate the structural aspects of CLAC, CD spectroscopy was performed on purified CLAC. The CD spectrum had a minor peak at 225 nm and a large minimum at 198 nm (Fig. 5B). The CD spectrum had a minor peak at 225 nm and a large minimum at 198 nm (Fig. 5B).

Purified CLAC Binds Aβ(1–40) and Aβ(1–42)—CLAC secreted into medium from HEK 293 cells stably expressing CLAC-P binds to aggregated Aβ(1–42), but not to soluble Aβ(1–42), in a solid-phase binding assay (18). Here, we use purified CLAC in a similar solid-phase assay. Immobilized Aβ(1–40) or Aβ(1–42) was incubated with purified CLAC, and bound CLAC was detected with anti-Myc antibody (directed against the C-terminal Myc/His tag of CLAC). A thioflavin T assay (26, 27) verified that the peptides were aggregated before coating onto the microtiter wells (data not shown). CLAC bound to immobilized Aβ(1–40) in a dose-dependent fashion (Fig. 6A), and a CLAC concentration of 10 nM was chosen for subsequent experiments. If the binding of CLAC to Aβ is based on ionic interactions, a perturbation of the binding depending on the NaCl concentration could be anticipated. Indeed, CLAC binding to Aβ was inversely related to NaCl concentration, and 0.5 μM NaCl added to TBS dramatically decreased binding to Aβ (Fig. 6B). No major differences in CLAC binding to immobilized Aβ(1–40) and Aβ(1–42) were observed (Fig. 6C), suggesting that the 2 extra residues in the Aβ(1–42) peptide are not crucial for binding to CLAC.

The interaction of CLAC with aggregates formed by two other amyloidogenic peptides was investigated in the solid-phase assay. One of the peptides was amylin, a 37-residue-long peptide, and the other was NAC, a 35-residue-long fragment of α-synuclein. Considerably less CLAC bound to amylin compared with Aβ (Fig. 6C), whereas CLAC bound to NAC with a similar affinity as for Aβ. The low affinity of CLAC for amylin aggregates was confirmed in a second set of experiments. Amylin and Aβ(1–40) aggregates were generated and mixed with an equal volume of medium from HEK 293 cells stably expressing CLAC-P-Myc/His. The samples were incubated and centrifuged, and the amount of CLAC in the pellet was determined by SDS-PAGE and Western blotting using anti-Myc antibody. About four times more CLAC was found in the Aβ pellet com-
triplicate experiments.

The interaction between purified CLAC and immobilized Aβ(1–40) was salt-dependent. CLAC was incubated with Aβ(1–40) in the presence of increasing concentrations of NaCl in TBS. At a concentration of 0.5 M NaCl in TBS, strong interaction between Aβ(1–40) and amylin. The interaction between CLAC and Aβ-(1–40) was obliterated. C, purified CLAC was incubated with immobilized Aβ(1–40), Aβ-(1–42), and the amyloidogenic amylin and NAC peptides. CLAC bound to Aβ-(1–40), Aβ-(1–42), and NAC with similar efficiency, whereas considerably less CLAC bound to amylin. D, conditioned medium from HEK 293 cells stably expressing CLAC-P-Myc/His was incubated with pre-aggregated Aβ-(1–40) and amylin in solution. After centrifugation, the amount of CLAC co-purifying with the respective peptide aggregates was quantified. Much less CLAC copurified with the amylin aggregates than with the Aβ-(1–40) aggregates. Error bars indicate means ± S.E. of triplicate experiments.

pared with the amylin pellet (Fig. 6D). Thus, two different assays showed that CLAC bound to aggregated Aβ, but not to aggregated amylin. The interaction of CLAC with Aβ was not restricted to only Aβ aggregates since similar binding was seen with NAC aggregates (Fig. 6C). Amylin does not contain any negatively charged residues, whereas Aβ and NAC contain several. However, negatively charged residues per se are not sufficient for CLAC binding to Aβ since CLAC binding to Aβ aggregates could not be competed with soluble Aβ-(1–28) (data not shown). Deglycosylation of CLAC using O-glycosidase, peptide N-glycosidase F, and neuraminidase had no effect on the binding affinity for Aβ aggregates (data not shown), suggesting that the interaction seen is not due to the carbohydrate groups on CLAC.

Aβ Interacts with a Motif in the NC2 Domain—To identify CLAC sequences important in mediating Aβ binding, we used the SPOTs method, a method well established for studying protein/protein interactions (28). Overlapping peptides corresponding to the sequences of the NC2–4 domains of CLAC-P were synthesized on a nitrocellulose membrane. The COL domains in CLAC were not synthesized since they are similar to those in collagen type XIII, and no studies have shown an interaction of collagen type XIII with amyloid plaques. Incubation of the membrane with radioactive Aβ-(1–40) generated a strong interaction between Aβ-(1–40) and peptides 6–11 on the SPOTs membrane (Fig. 7A). In the two most prominent spots, the common motif was the LIKRRLIK sequence, found at residues 181–188 in the NC2 domain of CLAC-P (Fig. 7B). The same results were obtained with another membrane probed with commercial iodinated Aβ-(1–42) (data not shown). No differences in the binding patterns between Aβ-(1–40) and Aβ-(1–42) were observed (data not shown), confirming that the very C-terminal residues of Aβ are not crucial for the binding of CLAC to Aβ.

Deletion or Substitution of the LIKRRLIK Sequence in CLAC Attenuates Aβ Binding—To further investigate whether the LIKRRLIK sequence is important for the interaction between CLAC and Aβ, three CLAC mutants were generated. One of the mutants lacks the LIKRRLIK sequence (ΔLIKRRLIK-CLAC) (Fig. 8A). This mutant showed a similar binding efficiency for heparin and eluted at the same NaCl concentration as wild-type CLAC (data not shown). All CLAC mutants were stably expressed in HEK 293 cells, and the purification scheme optimized for wild-type CLAC was applied to the purification of the
FIG. 8. Mutagenesis of the LIKRRLIK motif in CLAC. A, wild-type CLAC (WT-CLAC) bound to immobilized Aβ(1–40) in a dose-dependent manner, whereas no binding was observed for ΔLIKRRLIK-CLAC. Error bars indicate means ± S.E. of triplicate experiments. B, purified CLAC and ΔLIKRRLIK-CLAC were incubated with pre-aggregated Aβ(1–40) in solution. After centrifugation, the amount of CLAC and mutant CLAC co-precipitating with Aβ aggregates was quantified. Much less ΔLIKRRLIK-CLAC coprecipitated (35% of wild-type CLAC) with Aβ aggregates. Error bars indicate means ± S.E. of triplicate experiments. C, shown is competition with the 12-mer peptide QQLIKRRLIKGD. Five nM CLAC was incubated with varying amounts of coated Aβ(1–40) (0.25, 0.5, and 1.0 μg) in the presence of increasing concentrations of the peptide. Error bars indicate means ± S.E. of triplicate experiments. D, left panel, CD spectroscopy was performed on wild-type CLAC (○) and ΔLIKRRLIK-CLAC (△). No differences in the CD spectra could be detected between the wild-type and mutant molecules. Right panel, shown is the pepsin cleavage of equal amounts of wild-type CLAC (lane 1) and ΔLIKRRLIK-CLAC (lane 2). The same pepsin-resistant fragments were generated. mdegs, millidegrees. E, upper panel, the binding of CLAC containing the collagen type XIII-derived VIKRRTFQ sequence instead of the LIKRRLIK motif was analyzed. The mutant molecule showed impaired binding to immobilized Aβ(1–40). The binding of ΔLIKRRLIK-CLAC is shown in comparison. Error bars indicate means ± S.E. of triplicate experiments. Lower panel, shown are the results from immunoblotting of wild-type CLAC, VIKRRTFQ-CLAC, and ΔLIKRRLIK-CLAC in the solid-phase assay. Quantification was carried out with ECL and a CCD camera. F, upper panel, the binding of CLAC lacking the positively charged cluster in the COL1 domain was analyzed. The mutant showed no impaired binding to immobilized Aβ(1–40) compared with wild-type CLAC. Lower panel, shown are the results from immunoblotting of wild-type CLAC and ΔKRGKRGRR-CLAC used in the assay. Quantification was carried out with ECL and a CCD camera.

Mutants. The purity of the mutants was estimated to be the same as for wild-type CLAC (data not shown). The binding of wild-type and mutant CLAC to immobilized Aβ(1–40) was monitored by the solid-phase assay. Whereas wild-type CLAC bound to immobilized Aβ(1–40) in a concentration-dependent manner, no significant binding could be observed for ΔLIKRRLIK-CLAC (Fig. 8A), suggesting that the LIKRRLIK sequence is intimately involved in the interaction with Aβ. In addition, the sedimentation assay was used to study the binding of CLAC or ΔLIKRRLIK-CLAC to pre-made Aβ(1–40) aggregates. Analysis of the pellet fraction by Western blot showed that ΔLIKRRLIK-CLAC bound less to Aβ aggregates; only ~35% of mutant CLAC compared with wild-type CLAC co-sedimented with Aβ(1–40) aggregates (Fig. 8B). Additional experiments showed 15 and 30% co-sedimentation of mutant CLAC compared with wild-type CLAC (data not shown). To further investigate the importance of the LIKRRLIK sequence, a competition assay was performed using a peptide with the sequence QQLIKRRLIKGD. Co-incubation of CLAC and aggregated Aβ with the QQLIKRRLIKGD peptide showed a dose-dependent inhibition of CLAC binding (Fig. 8C). No major differences in secondary structure between CLAC and ΔLIKRRLIK-CLAC could be detected by CD spectroscopy (Fig. 8D). In addition to CD analysis, pepsin cleavage of ΔLIKRRLIK-CLAC was performed, and no differences between wild-type CLAC and ΔLIKRRLIK-CLAC could be observed in the fragment pattern after pepsin digestion (Fig. 8D), further indicating that ΔLIKRRLIK-CLAC has no major structural changes. Thus, the loss of binding is not due to a deletion-dependent major structural change in the conformation of CLAC.

In the second mutant, the LIKRRLIK sequence is replaced with VIKRRTFQ (VIKRRTFQ-CLAC), a sequence located in the NC2 domain of collagen type XIII (33). Interestingly, in the solid-phase assay, the VIKRRTFQ-CLAC mutant showed intermediate binding to immobilized Aβ(1–40) (Fig. 8E). This binding might be mediated by the sequence IKRR, which is identical to the wild-type sequence. The third mutant generated lacks a 9-residue-long charged motif in the COL1 domain of CLAC (ΔKRGKRGRR-CLAC) (Fig. 8F). The binding efficiency of this mutant for Aβ was the same as that of wild-type CLAC (Fig. 8F), suggesting that the major binding site for Aβ is the LIKRRLIK sequence.

**DISCUSSION**

The type II transmembrane protein CLAC-P/collagen XXV is exclusively expressed in neurons (18). The extracellular CLAC
domain, which is suggested to be shed via a furin-like cleavage of CLAC-P, co-localizes with senile plaques in AD and Down’s syndrome brains (18). The influence of CLAC with regard to plaque formation is unknown; CLAC might play a role in Aβ polymerization, protect senile plaques from degradation, or be an innocent bystander. This study presents findings obtained from the characterization of purified recombinant CLAC. We determined the exact cleavage site generating the soluble CLAC ectodomain and have shown that CLAC is glycosylated and hydroxylated and adopts a pepsin-resistant triple-helical structure. We also explored the interaction between CLAC and Aβ in vitro and reported the identification of an Aβ-binding motif in the NC2 domain of CLAC.

Expression of recombinant collagens is usually performed in insect cells, but due to the low endogenous activity levels of prolyl 4-hydroxylase, coexpression of this enzyme is commonly used in this system (34). Here, we used HEK 293 cells to express the precursor protein CLAC-P, a system previously reported to produce correctly folded recombinant collagens (35). A purification method was developed in which CLAC was isolated and purified to homogeneity from culture medium from stably expressing HEK 293 cells by cation-exchange and nickel affinity chromatography. Purified CLAC migrated with a molecular mass of ~75 kDa, which is larger than the molecular mass predicted from the cDNA sequence. Aberrant migration on SDS-polyacrylamide gel is a common feature of collagens and the high content of imino groups in collagens. Recombinant CLAC also differs in size from CLAC isolated from human AD brain, which migrates as 50- and 100-kDa protein species (the latter probably representing the dimer) (18, 19). These species might be truncated variants of CLAC as a result of alternative splicing of its transcripts, a well-characterized feature of the collagen type XIII gene (37). Another possibility is that CLAC is proteolytically degraded before or after their deposition in plaques.

It was crucial to obtain correctly folded CLAC since the studies herein were aimed at identifying and studying possible binding site(s) between CLAC and Aβ. We have shown by CD spectroscopy and protease resistance assays that recombinant CLAC folds into a triple-helical structure, a structure specific for the collagen family. Collagen chain association has been studied most extensively for fibrillar collagens. Recent studies have suggested two separate motifs important for the correct folding of collagen type XIII: one region in the NC1 domain and one in the NC3 domain (38). These motifs are conserved in collagen types XXIII and XXV and have therefore been suggested to be of importance also for these transmembrane collagens (38, 39). In accordance with a previous report (18), CLAC formed dimers and trimers even though the splice variant expressed in this study lacks parts of the sequences of the COL3 and NC4 domains (residues 589–640, according to the numbering of GenBankTM/EBI accession number AF293341). Multimer formation has also been observed with a COL3 and NC4 domain-truncated collagen XIII protein, indicating that the C-terminal domains of these collagens are not critical for chain association (40). The majority of CLAC isolated from human AD brain is in a monomeric or dimeric form; a trimeric form is rarely observed.

Several heparan sulfate proteoglycans have been found to co-deposit with senile plaques and to interact with Aβ (12–14). Of particular interest is the binding of CLAC to heparin, suggesting that CLAC might have the potential to associate with other matrix or membrane-bound heparan sulfate proteoglycans. This interaction is probably mediated through the interaction between the positively charged clusters in the COL1 domain of CLAC and the negatively charged sulfated glycosaminoglycan chains of heparin. The functions of many of the recently identified transmembrane collagens, including collagen types XIII and XXV, are unknown. Speculations exist as to whether collagen XIII is involved in cell/cell contact and cell/matrix adhesion (41), a function that can also be attributed to CLAC. Since CLAC is exclusively expressed in neurons, it might function as a brain-specific collagen that plays a role in the adhesion of neurons to the extracellular brain matrix. Although no RGD motifs exist in the CLAC sequence, it has numerous KGD sequences that could mediate integrin binding (42).

A recent study showed that CLAC binds to Aβ-(1–42) aggregates in an in vitro solid-phase binding assay (18). The findings of the present study complement this work and explore the CLAC/Aβ interaction in more detail. We have shown that purified CLAC binds to Aβ-(1–40) with a similar affinity as for Aβ-(1–42). This implies that the 2 extra residues in Aβ-(1–42) are not critical for Aβ binding to CLAC. CLAC bound to aggregated Aβ, but not to its soluble form; and thus, it is possible that CLAC interacts with the fibrillar structure per se. To investigate whether CLAC binds to amyloid in general, we used aggregates formed by the amyloid peptide amyl in two parallel approaches: a solid-phase and a solution-based method. CLAC binding to amyl was markedly reduced compared with Aβ in both methods (Fig. 6, C and D). CLAC interaction with NAC was also investigated, and the binding observed was very similar to that with Aβ (Fig. 6C). Both Aβ and NAC contain negatively charged residues, whereas no such residues are present in amyl in. Therefore, we suggest that not only a fibrillar structure, but also negatively charged residues are of importance for the binding of CLAC.

SPOTs synthesis revealed an Aβ-binding site within the NC2 domain, close to the N terminus of CLAC. The ΔLIKRRLIK-CLAC mutant completely lost its ability to interact with Aβ, and CLAC binding to Aβ was competed by the addition of the QQLIKRRIKGD peptide (Fig. 8, A and C). Replacing the LIKRRLIK sequence with the VIKRRTFQ sequence found in collagen XIII resulted in a CLAC molecule showing intermediate binding to Aβ (Fig. 8E). We suggest that the IKRR sequence is important for this binding. It has been noted that a positively charged cluster located in the COL1 domain, resembling the LIKRRLIK sequence, affects the CLAC/Aβ interaction (43). These observations indicate that there might exist more than one Aβ-binding site in CLAC; and therefore a third mutant (ΔKRGKRGRR-CLAC) lacking this highly charged cluster in the COL1 domain was generated. This mutant did not show reduced Aβ binding (Fig. 8F), suggesting that the LIKRRLIK sequence is the major Aβ-binding motif in CLAC. Additional experiments are required to determine whether the Aβ-binding motif found herein, the LIKRRLIK sequence, is the only binding site for Aβ.

The complement protein C1q, which is co-localized with Aβ depositions in AD brain (44), activates the complement pathway, leading to an inflammatory response, and dramatically enhances the aggregation of Aβ in vitro (45). It is interesting to note that the N-terminal region of C1q contains a cluster of 5 positively charged residues resembling the LIKRRLIK sequence in CLAC. This motif in C1q is critical for the interaction with Aβ, which is mediated by negatively charged residues in the N terminus of Aβ (Aβ-(1–11)) (46). It is tempting to speculate that a similar ionic interaction might exist between CLAC and Aβ since this interaction is inversely related to NaCl concentrations. Therefore, Aβ-(1–11) is considered as a likely candidate for the CLAC-binding site. Further studies are needed to address these possibilities.

In summary, we have devised a protocol for the expression...
and purification of CLAC. The protein obtained was of high purity and showed the predicted biochemical/biophysical properties. Two independent assays showed that purified CLAC binds to aggregated Aβ-(1–40) and Aβ-(1–42) with similar affinity, but not to all amyloid fibrils. A charged sequence in the NC2 domain of CLAC mediates the binding to Aβ in a salt-dependent manner. The identification of this sequence may be useful for designing compounds aimed at modulating the CLAC/Aβ interaction.

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