CLAC Binds to Amyloid β Peptides through the Positively Charged Amino Acid Cluster within the Collagenous Domain 1 and Inhibits Formation of Amyloid Fibrils

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Alzheimer’s disease (AD)1 is an elderly onset neurodegenerative disease causing dementia that is pathologically characterized by a massive accumulation of amyloid deposits in senile plaques or cerebrovasculature and of tau-rich neurofibrillary tangles or cerebrovasculature and of tau-rich neurofibrillary tangles. We previously showed that CLAC binds to the fibrillized form of Aβ in vitro, although the mechanism and the subdomains that mediate interaction of CLAC with Aβ as well as the effect of binding of CLAC on amyloid fibril formation remain unknown. Here we show that the collagenous domain 1 of CLAC, which is rich in positively charged amino acid residues, mediates its interaction with Aβ and that this binding is mediated by an electrostatic interaction and requires formation of the triple helix structure of CLAC. The soluble form of CLAC purified from the media of cells transfected with CLAC-P inhibited fibrillation of Aβ in vitro, especially in its elongation phase. These results suggest the anti-amyloidogenic roles of CLAC in the pathophysiology of Alzheimer’s disease.

CLAC (collagenous Alzheimer amyloid plaque component) is a proteolytic fragment derived from a novel membrane-bound collagen, CLAC-P/collagen type XXV, that deposits in senile plaques associated with amyloid β peptides (Aβ) in the brains of patients with Alzheimer’s disease. We previously showed that CLAC binds to the fibrillized form of Aβ in vitro, although the mechanism and the subdomains that mediate interaction of CLAC with Aβ as well as the effect of binding of CLAC on amyloid fibril formation remain unknown. Here we show that the collagenous domain 1 of CLAC, which is rich in positively charged amino acid residues, mediates its interaction with Aβ and that this binding is mediated by an electrostatic interaction and requires formation of the triple helix structure of CLAC. The soluble form of CLAC purified from the media of cells transfected with CLAC-P inhibited fibrillation of Aβ in vitro, especially in its elongation phase. These results suggest the anti-amyloidogenic roles of CLAC in the pathophysiology of Alzheimer’s disease.

1 The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid β; CLAC, collagenous Alzheimer amyloid plaque component; sCLAC, soluble CLAC; CLAC-P, CLAC precursor; α1M, α1-microglobulin; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; mt, mutant; wt, wild type; thiO, thioplatin.

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To test this hypothesis by in vitro experiments, we studied the effects of sCLAC on Aβ fibrillation in vitro and examined the mechanism as well as the subdomains that mediate interaction of sCLAC with Aβ. We found that sCLAC in its proper triple helix form binds to Aβ fibrils through the positive charge linked to early onset familial AD increase the production of Aβ42 (6–8). These findings collectively suggest that aggregation and deposition of Aβ are closely linked to the pathogenesis of AD.

A number of non-Aβ proteinaceous components, e.g. apolipoprotein E (apoE), α1-antichymotrypsin, α2-macroglobulin, complement component C1q, amyloid P component, have been identified associated with senile plaque amyloids (9), some of which have been shown to affect fibrillation of Aβ (10). Genetic polymorphism of apoE, especially the e4 genotype, is known as the major genetic risk factor of AD (11), and accumulating evidence from experimental studies suggests that apoE may affect the fibrillation and deposition of Aβ, thereby leading to AD; ablation of apoE gene reduced Aβ deposition in transgenic mice overexpressing human βAPP, whereas further transgenic supplementation of human apoE e4 gene accelerated deposition of β-sheet-rich Aβ (12). In vitro studies, however, showed that apoE also has an inhibitory effect on fibrillation of Aβ (13, 14), implicating apoE in various aspects of β-amyloid formation in AD. These previous results emphasize the compelling need for the analysis of effects of other non-Aβ components of senile plaques on the fibrillation and deposition of Aβ.

We have recently identified a novel collagenous protein as a component of senile plaque amyloid and designated it as CLAC (collagenous Alzheimer amyloid plaque component) (15). CLAC is derived from its precursor CLAC-P/type XXV collagen, a member of collagenous transmembrane proteins (16), specifically expressed in neurons. We also showed that the ectodomain of CLAC-P is shed by furin convertase and secreted as a soluble form of CLAC (sCLAC) (15). Immunohistochemical analysis of postmortem human brains revealed a unique pattern of CLAC deposition in AD and Down’s syndrome brains (17); CLAC was negative in Aβ42-positive, Aβ40-negative pure diffuse plaques that are considered to be the earliest form of Aβ deposition, whereas a fraction of primitive-type senile plaques became CLAC-positive at a relatively early stage of plaque maturation as the fibrillation of Aβ progresses. Notably, mature plaques that appear at later stages were Aβ40/thioflavin S-positive but remained CLAC-negative (17). These findings suggested the possibility that CLAC may play an inhibitory role in the deposition of senile plaque amyloid by binding to early Aβ deposits and preventing further incorporation of Aβ peptides to develop β-sheet-rich, mature senile plaques.
cluster within the collagenous domain 1 and that binding of sCLAC inhibits fibrillation of Aβ in the elongation phase of amyloid fibril formation, providing further support to the inhibitory role of CLAC in β-amyloidogenesis.

Materials and Methods

Reagents and Chemicals—Synthetic Aβ(1–40) and Aβ(1–42) peptides were purchased from Peptide Institute, Inc. (Osaka, Japan). Aβ(1–40) and Aβ(1–42) were used in experiments shown in Fig. 5 as well as Aβ(1–28), Aβ(1–16), Aβ(1–17–22), and Aβ(1–22–28) were purchased from AnaSpec (San Jose, CA). Heparin sodium salt was purchased from Sigma. Human recombinant apoE (apoE3) was purchased from Wako Pure Chemical (Osaka, Japan). Human α1-microglobulin was purchased from DAKO (Denmark).

DNA Constructs—Human CLAC-P cDNA (15) was subcloned into a mammalian expression vector pcDNA3.1 (Invitrogen). Mutations were introduced into the CLAC-P cDNAs by in vitro site-directed mutagenesis using the following oligonucleotides: NC1cm 5′-GACCTGAGGGGAGAAGGCCGTAAAGGATCACC-3′ (forward), 5′-GGCGATTTCTTCATAGCCGGCTGCAC-3′ (reverse); NC3cm 5′-CTCCAGGAA ACTTGTAGGAAAGAGCAACCCAC-3′ (1st step forward), 5′-GGCTGTTCTC CTCCTGAATGCTTCTTGGAG-3′(1st step reverse), 5′-GGCTAATCAGAA GAGCCTACGAC CC-3′ (2nd step forward), 5′-GCCTCAGGGAGAT GAGGTCCTTTC TTAGGGCTCC-3′ (2nd step reverse); COL3-1mt 5′-GCCCGTGGGCCCCTAGTTCCTG-3′ (forward), COL3-2mt 5′-GGACCGGTCCTGGGGGCTTACGAGGAGCAGGGTTCACCTTGGTGGGGCCTGGCGGACCTGGGGGCCCTGGGAGGGCCTGC-3′ (reverse), COL3-2mt 5′-GAGGACATTGGGGCCACCGTGGCTGGACCGGCGACCTC-3′ (forward), COL3-3mt 5′-GGCCCTGCTTTTTCTCCAGGGATCCCCCGGTGAC-3′ (reverse); COL3-1mt 5′-GCCCGTGGGCCCCTAGTTCCTG-3′ (forward), COL3-2mt 5′-GGACCGGTCCTGGGGGCTTACGAGGAGCAGGGTTCACCTTGGTGGGGCCTGGCGGACCTGGGGGCCCTGGGAGGGCCTGC-3′ (reverse); COL3-3mt 5′-GGCCCTGCTTTTTCTCCAGGGATCCCCCGGTGAC-3′ (reverse); COL4 5′-GGTGTTCTTGCTTTCTCCAGGGATCCCCCGGTGAC-3′ (forward); COL4 5′-GGTGTTCTTGCTTTCTCCAGGGATCCCCCGGTGAC-3′ (reverse); CO2 5′-GAGGAAACCAGGGTGAAGCTTCTAAGGAACCTCCTGTTC-3′ (forward), 5′-GACATACGTCCCTCAGGCTTTCCTCCGGCCTGGC-3′ (reverse); COL4 5′-GAGGAAACCAGGGTGAAGCTTCTAAGGAACCTCCTGTTC-3′ (forward), 5′-GACATACGTCCCTCAGGCTTTCCTCCGGCCTGGC-3′ (reverse); COL2 5′-GAGGAAACCAGGGTGAAGCTTCTAAGGAACCTCCTGTTC-3′ (forward), 5′-GACATACGTCCCTCAGGCTTTCCTCCGGCCTGGC-3′ (reverse).

Antigen and Antibody Preparations—Anti-NC3 and anti-NC4 polyclonal antibodies were raised in rabbits against synthetic peptides corresponding to the NC3 (DYNGNLHEALQRITC) and NC4 (LGPDG...) regions of CLAC-P as described (15). SDS-PAGE was performed as described (8). Stable cell lines were generated by transfection of cDNAs using LAS-1000plus (FUJIFILM) as described (8).

In Vitro Binding Assay—In vitro binding assay for sCLAC and fibrillized Aβ or heparin immobilized on a solid phase was performed as described (15). Briefly, a 50-μl aliquot of 100 μg/ml heparin, bovine serum albumin (a negative control), and purified synthetic Aβ(1–42) (Peptide Institute, Inc.) was allowed to dry on wells of enzyme-linked immunosorbent assay plates, blocked for 1 h with Block Ace (Snow Brand, Sapporo, Japan), and washed with PBS containing 0.05% Tween 20. Microplate wells were then incubated with conditioned media of HEK293 cells stably transfected with wild-type or mutant CLAC-P cDNAs for 1 h, washed with PBS containing 0.05% Tween 20, and then reacted with anti-NC4 antibody for 1 h. After incubation with a horseradish peroxidase-tagged secondary antibody, a substrate was reacted with anti-NC4 antibody for 1 h. After incubation with a horseradish peroxidase-tagged secondary antibody, a substrate was added. Fluorescence levels of samples were assayed using fluorescence spectrophotometer F2500 (Hitachi) at 543 nm and λex of 484 nm, respectively, and the fluorescence level of a 500-μl aliquot of 3 mm thioflavin T in 0.1 M glycine-NaOH (pH 8.5) buffer prechilled at 4 °C was used as a standard. Negative-stain electron microscopy—Samples were spread on 400-mesh collagen-coated grids and then negatively stained with 2% phosphotungstic acid (pH 7.0) and viewed in an electron microscope (JEOL 1200EXII) as described (19). Negative-stain immunoelectron microscopy was performed as described (19). Briefly, samples were spread on grids and blocked by 1% gelatin containing PBS for 30 min and then incubated with anti-CLAC-P antibody for 1 h. After washing, the grids were incubated by anti-rabbit IgG secondary antibody conjugated with 10-nm colloidal gold for 1 h and negatively stained and viewed by electron microscopy as above.

Results

Collagenous Triple-helix Structure of CLAC Is Indispensable to Its Interaction with Fibrillized Aβ—CLAC-P is a transmembrane protein with a type II orientation (Fig. 1A). We previously reported that CLAC-P is cleaved at Arg-112—Glu-113 by furin convertase, thereby liberating the extracellular domain as a soluble/secrected form of CLAC-P (sCLAC) (15). CLAC-P harbors three Gly-X-Y collagenous repeat motifs and forms a homotrimeric structure under non-reducing conditions (15). We examined whether sCLAC retains the triple-helix structure by a trypsin digestion assay. Native collagens with a triple-helix structure are resistant to digestion by authentic proteases including trypsin or pepsin, whereas heat-denatured collagen at >40 °C is susceptible to those proteases (18, 20). We heated conditioned media of HEK293 cells stably transfected with human CLAC-P containing sCLAC at various temperatures between 25–60 °C and then digested them by trypsin and found that sCLAC remained resistant to trypsin digestion by preheating at 45 °C, whereas it became labile at 50 °C (Fig. 1B). We previously reported that sCLAC is bound specifically to fibrillized form of Aβ by an in vitro solid-phase Aβ binding assay (15). To examine whether the triple-helix structure of sCLAC is required for the binding between sCLAC and fibrillized Aβ, we preheated conditioned media containing sCLAC at 25–60 °C and subjected them to the in vitro Aβ binding assay.

Purification of sCLAC—Purification of sCLAC was summarized in the flow chart in Fig. 6A. Briefly, conditioned media of HEK293 cells stably expressing human CLAC-P were initially applied to a DEAP-cellulose column (DE52, Whatman), and the flow-through fractions were then applied to a heparin-Sepharose column (Amersham Biosciences) and eluted with phosphate buffer containing 1 mM NaCl. The eluates were dialyzed against PBS, concentrated, and further separated by reverse phase HPLC on an Aquapor RP300 column with a linear gradient of 0–64% acetonitrile in 0.1% trifluoroacetic acid. Purified sCLAC in acetonitrile solution was evaporated and dissolved in 1 mM HCl at 0.1 mg/ml.

In Vitro Aβ Fibrillation and Thioflavin T Fluorescence Assays—Synthetic Aβ(1–40) or Aβ(1–42) peptides were solubilized in PBS including 2% Me_SO at 100 μg/ml and then filtered through a 0.22-μm pore filter immediately before use. Purified sCLAC or α1-microglobulin (α1Mg) was added into 50-μl aliquots of Aβ(1–42) solution at molar ratios of 100/1 (Aβ/sCLAC or α1Mg) and incubated at 37 °C for 0, 0.5, 1, 2, 4, 8, 24 h. After incubation, the aliquots were immediately put on ice, and 500-μl aliquots of 3 mM thioflavin T in 0.1 M glycine-NaOH (pH 8.5) buffer prechilled at 4 °C were added purified proteins—seeds (molar ratio of Aβ(1–40)/(Aβ(1–40)) at 1:100/1) at 37 °C with continuous mixing as described above. To selectively examine the elongation phase of amyloid fibril formation by bypassing the nucleation phase, purified sCLAC, apoE, or α1Mg (molar ratio of Aβ(1–40) peptides = 250/1) were incubated with Aβ(1–40) seeds (molar ratio of Aβ(1–40)/fibrillized Aβ(1–42) = 100/1) and then incubated at 37 °C for 0, 1, 2, 4, 8, 24, or 48 h and immediately analyzed as above.

Negative Stain Electron Microscopy—Samples were spread on 400-mesh collagen-coated grids and then negatively stained with 2% phosphotungstic acid (pH 7.0) and viewed in an electron microscope (JEOL 1200EXII) as described (19). Negative-stain immunoelectron microscopy was performed as described (19). Briefly, samples were spread on grids and blocked by 1% gelatin containing PBS for 30 min and then incubated with anti-CLAC-P antibody for 1 h. After washing, the grids were incubated by anti-rabbit IgG secondary antibody conjugated with 10-nm colloidal gold for 1 h and negatively stained and viewed by electron microscopy as above.

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sCLAC preheated at <45 °C was bound to Aβ, whereas sCLAC denatured at >50 °C failed to interact with fibrillized Aβ (Fig. 1C). This strongly suggested that the preservation of the collagenous triple-helix structure of sCLAC is the prerequisite for its interaction with Aβ fibrils.

CLAC-P harbors two highly conserved, α-helical coiled-coil domains that are composed of repeats of heptad sequences (abcdefg; hydrophobic amino acid residues occur at positions a and d) that are shown to be important for the assembly of a set of membrane-bound collagens including type XIII collagen, a homologue of CLAC-P (type XXV collagen (21), within the NC1 and NC3 domains (Fig. 2A). Moreover, it has been shown that type XIII collagen fails to form a triple helix and becomes misfolded when the NC1 coiled-coil domain is deleted (22). We generated three mutant (mt) CLAC-P cDNAs (NC1ccmt, NC3ccmt, and NC1/NC3ccmt) in which the hydrophobic amino acid residues within the coiled-coil domains are replaced with lysine and stably expressed them in HEK293 cells. NC1ccmt CLAC-P was moderately sensitive to trypsin digestion, suggesting a partial impairment in the triple-helix structure, whereas the NC3ccmt and especially the NC1/NC3ccmt, CLAC-P, were highly sensitive to trypsin digestion without prior denaturation, indicating the lack of the triple-helix formation (Fig. 2B). We then tested the relationship between triple-helix formation of CLAC-P and sCLAC/Aβ binding by the in vitro Aβ binding assay. Binding of fibrillized Aβ relative to that with wild-type (wt) CLAC-P was slightly reduced in NC1ccmt CLAC-P, whereas it was significantly impaired with NC3ccmt CLAC-P and completely lost with NC1/NC3ccmt sCLAC polypeptides (Fig. 2C). These results clearly indicate that the collagenous triple-helix structure of sCLAC is indispensable to its interaction with Aβ fibrils.

The Binding between Fibrillized Aβ and sCLAC Is Mediated by Electrostatic Interaction and Is Competed by Heparin—We previously reported that the interaction between fibrillized Aβ and sCLAC is blocked by a high concentration of NaCl (15). To examine the effects of ionic strength in more detail, we dialyzed conditioned media containing sCLAC against 10 mM phosphate buffer followed by the addition of NaCl at concentrations of 0–1 M and subjected them to the in vitro Aβ binding assay and found that the presence of high concentrations of NaCl inhibited the interaction between fibrillized Aβ and sCLAC (IC50 = 0.73 M) (Fig. 3A). The possibility that Aβ immobilized on wells were detached by a high concentration of NaCl was excluded by probing the plates by an anti-Aβ antibody (data not shown). These data further confirm that sCLAC is bound to fibrillized Aβ through an electrostatic interaction.

We next investigated the interaction between sCLAC and heparin. sCLAC harbors one of the predicted binding consensus motifs for heparin (XBBXXBX or XBBBXBX, where B and X are basic and hydrophobic residues, respectively) (23) at HRRKLIKSG in the NC2 domain. In addition, sCLAC has four positively charged amino acid cluster regions within its collagenous domains (Fig. 4A), where positively charged amino acid residues are frequently located at X and/or Y positions in the Gly-Y-X collagens. These domains are predicted to constitute a cluster of positive charges when CLAC-P or sCLAC form a native triple-helix structure. Such positive charge clusters have been implicated in the binding between collagen and heparin (24, 25). Taken together with the previous findings that type XIII and XXIII collagens bind to heparin (26, 27), we suspected if one or the other of the four positively charged amino acid cluster regions of CLAC-P may serve as binding domains for heparin. We examined the binding of sCLAC and heparin by an in vitro binding assay, coating heparin onto microwell plates and found that sCLAC and heparin bind each other (Fig. 3B) and that the interaction is completely inhibited by the addition of 1 mM NaCl (data not shown). These results suggested that sCLAC also was bound to heparin by an electrostatic interaction. To examine if heparin competes for...
the binding domain of sCLAC with fibrillized Aβ, we added different concentrations of heparin (0–1 mg/ml) to the conditioned media containing sCLAC in the \textit{in vitro} Aβ binding assay and confirmed that heparin inhibits the binding between sCLAC and fibrillized Aβ in a dose-dependent manner (Fig. 3C). These results strongly suggest that fibrillized Aβ and heparin interacts with an identical subdomain in sCLAC.

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**Fig. 2.** Coiled-coil domains in NC1 and NC3 domains of CLAC-P are necessary for the triple-helix formation and interaction with fibrillized Aβ. \(A\), alignments of amino acid sequences of the coiled-coil domains in juxtamembrane (NC1) and NC3 domains of CLAC-P and other membrane-bound collagens. Hydrophobic residues in positions a and d of the coiled-coil heptad repeats are shaded. The locations of residues substituted to Lys in the coiled-coil mutant (cc mt) CLAC-P (NC1ccmt and NC3ccmt) are outlined in black. \(h\), human; \(m\), mouse; TMD, transmembrane domain. \(B\), tryptic digestion of coiled-coil mutant sCLAC. Total levels of proteins subjected to the assay are shown in the lane trypsin (–). Samples were analyzed by immunoblotting with anti-NC4 after preincubation at temperatures of 20–55 °C (shown above the lanes) and subsequent digestion by trypsin. CLAC-P\textsubscript{wt}, sCLAC derived from wt CLAC-P; \(C\), \textit{in vitro} binding assay of sCLAC with fibrillized Aβ to wt or coiled-coil mt sCLACs. Relative optical densities of immunoreactions of sCLAC bound to Aβ (relative to that of wt CLAC as 1.0) are shown. The mean ± S.D. of optical densities in three independent experiments is shown. \(p < 0.05\) (*) and \(p < 0.001\) (**), respectively, by Student’s \(t\) test.

**Fig. 3.** sCLAC binds to fibrillized Aβ and heparin by electrostatic interaction. \(A\), \textit{in vitro} binding assay of sCLAC with fibrillized Aβ in the presence of various concentrations of NaCl. The relative optical densities (relative to that in the absence of NaCl (0 M) as 1.0; mean ± S.D. in three independent experiments) are shown. The IC\(_{50}\) value was \(-0.73\) M. \(B\), \textit{in vitro} binding assay of sCLAC with heparin. Optical densities for sCLAC binding to heparin immobilized onto microtiter plates (mean ± S.D. in six independent experiments) are shown. *, \(p < 0.05\) by Student’s \(t\) test. BSA, bovine serum albumin. \(C\), the binding of sCLAC with fibrillized Aβ was inhibited by the addition of various concentrations of heparin (0–1.0 mg/ml). Relative optical densities (relative to that without the addition of heparin as 1.0; mean ± S.D. in three independent experiments) are shown.

The Positively Charged Amino Acid Cluster in the COL1 Domain Is Crucial for the Interaction of sCLAC with Fibrillized Aβ—We found that the preservation of the collagenous triple-helix structure of sCLAC is required for its interaction with fibrillized Aβ, suggesting that Aβ fibrils recognize the three-dimensional structure, not simply the primary structure, of sCLAC. Furthermore, the heparin binding region of sCLAC seems to overlap with that for Aβ fibrils. These findings led us to hypothesize that one or more of the positively charged amino acid cluster regions within the collagenous domain may especially be important for the interaction of sCLAC with fibrillized Aβ (Fig. 4A). We then constructed five mt CLAC-P cDNAs (COL1\textsubscript{mt}, COL2\textsubscript{mt}, COL3-1\textsubscript{mt}, COL3-2\textsubscript{mt}, and COL1/3-2 double\textsubscript{mt}) in which the positively charged amino acid residues in the X and/or Y positions of the Gly-X-Y collagenous repeats were replaced with proline, the latter being the most common residue at the X or Y positions in collagens and contributing to the stability of the triple-helix structure. We stably expressed these mt CLAC-P cDNAs in HEK293s cells and confirmed that
the mt sCLAC proteins also were secreted into culture media (Fig. 4B), forming an intact triple-helix structure as determined by trypsin digestion (Fig. 4C). COL1mt, COL2mt, COL3-1mt, COL3-2mt, or COL1/3-2 double mt CLAC-P, wt or mt CLAC-P polypeptides are detected at ~70–80 kDa showing similar intensities, with slight variations in migration positions. C, trypsin digestion assays of wt and mt sCLAC recovered in conditioned media of HEK293 stable cells in B. Note that all mt sCLAC exhibited resistance to trypsin in a similar manner to wt sCLAC, suggesting proper trimer formation of mt sCLAC polypeptides. D, in vitro binding assays of wt or mt sCLAC with fibrillized Aβ. Binding of wt, COL1mt, COL2mt, COL3-1mt, COL3-2mt, or COL1/3-2 double mt sCLAC with fibrillized Aβ at different NaCl concentrations were monitored by the in vitro assay and plotted as ratios relative to the optical densities of immunoreactions for each mt sCLAC in the absence of NaCl as 1.0. Mean ± S.D. of optical densities in four independent experiments are shown. E, comparison of the binding of wt and COL1mt sCLAC with fibrillized Aβ at 131 mM NaCl (normalized by the levels of sCLAC in media). The amount of COL1mt sCLAC bound to fibrillized Aβ as detected by anti-NC4 antibody was ~20% that of wt sCLAC. The optical density with wt sCLAC was normalized to 1.0, and the mean ± S.D. in three independent experiments are shown.

Considering these differences in the reactivity of detector antibodies in the in vitro Aβ binding assay, we have chosen to compare the binding affinities of mt sCLAC to fibrillized Aβ as the relative strength of the ionic interactions in each mutant. To this end we dialyzed conditioned media containing wt or mt sCLAC against 10 mM phosphate buffer and then added NaCl to final concentrations of 0–1 M and subjected them to the in vitro Aβ binding assay. The interaction between wt sCLAC and Aβ was inhibited by the addition of >0.5 M of NaCl (Fig. 3, A and D) and COL2mt, COL3-1mt, or COL3-2mt sCLAC showed almost similar inhibition profiles of Aβ binding to that with wt sCLAC/Aβ Binding and Inhibition of Amyloid Fibril Formation
sCLAC (Fig. 4D). In sharp contrast, the interactions of COL1mt as well as the COL1/3-2 double mt sCLAC with fibrillized Aβ were inhibited by lower concentrations of NaCl compared with that of wt sCLAC (Fig. 4D). We further compared the absolute levels of sCLAC binding with fibrillized Aβ in wt and COL1mt sCLAC, which were confirmed to react at similar intensities with anti-NC3 or NC4 CLAC-P antibodies, and demonstrated that the binding of COL1mt sCLAC with Aβ is decreased (Fig. 4E). These data strongly suggested that the positively charged amino acid cluster region in the COL1 domain plays a crucial role in the binding to fibrillized Aβ.

Fibril Formation of Aβ Is Required for Its Interaction with sCLAC—We previously showed that CLAC-P and sCLAC specifically bind to a fibrillized form of Aβ (15). To locate the binding domain to sCLAC within Aβ, we used a series of synthetic Aβ fragment peptides (Fig. 5A). We preincubated Aβ fragment peptides to induce fibrillation and preabsorbed the conditioned media containing sCLAC with the peptides. Thus preabsorbed media were subjected to the in vitro Aβ binding assay coating Aβ-(1–42) on the solid phase. Preabsorption by Aβ-(1–42) or Aβ-(1–40) inhibited the interaction of residual sCLAC with fibrillized Aβ to a similar extent, whereas the interaction was not significantly inhibited by preabsorption with Aβ-(1–28), (1–16), (17–42) or Aβ-(12–28) (Fig. 5B). To evaluate the extent of fibril formation of Aβ fragment peptides, we quantitatively examined the β-sheet contents by thioflavin T (thioT) fluorescence, a fluorescent small molecule that binds to β-sheeted structures (28), and found that Aβ-(1–28), Aβ-(1–40), or Aβ-(17–42) showed increased thioT fluorescence reflecting formation of β-sheet rich fibrils, whereas other fragment peptides did not (Fig. 5C). Formation of fibrils was confirmed by negative-stain electron microscopy (data not shown). These results indicate that the fibril formation of Aβ is the prerequisite for interaction of Aβ with sCLAC. Furthermore, the binding domain of Aβ with sCLAC was estimated to be located within the N-terminal 16 amino acid residues because fibrillized Aβ-(17–42) did not bind sCLAC.

Purification of sCLAC and Binding to Fibrillized Aβ—To gain insights into the effects of sCLAC on Aβ fibril formation, we purified sCLAC from conditioned media of HEK293 cells stably expressing CLAC-P. Conditioned media were sequentially purified by a DEAE-cellulose column, heparin-Sepharose column, and reverse-phase HPLC (Fig. 6A). sCLAC was purified to a near homogeneity in fractions after separation by reverse-phase HPLC as determined by protein silver staining (Fig. 6B). To test whether purified sCLAC retained affinity with fibrillized Aβ, we examined the binding of purified sCLAC to fibrillized Aβ by the in vitro Aβ binding assay. Purified sCLAC showed affinity to bind fibrillized Aβ at ~40% of those in crude conditioned media (Fig. 6C), and negative-stain immunoelectron microscopy showed that purified sCLAC directly binds to prefibrillized Aβ fibrils (Fig. 6D).

sCLAC Inhibits Fibrillation of Aβ-(1–42) in Vitro—To verify the effects of sCLAC on fibrillation of Aβ, we incubated Aβ-(1–42) (at 0.1 mg/ml) alone or together with purified sCLAC or α1mG at a molar ratio of Aβ-(1–42)/sCLAC or α1mG at 100/1 and examined the extent and time course of Aβ fibrillation by thioT fluorescence. Purified sCLAC decreased fibril formation of Aβ-(1–42), whereas α1mG had no effects on its fibrillation (Fig. 7A). Electron microscopy showed the formation of Aβ fibrils by incubation of Aβ-(1–42) for 8 h (Fig. 7B), whereas coincubation of Aβ-(1–42) with purified sCLAC abolished formation of fibrils at the same incubation period (Fig. 7B). To rule out the possibility that sCLAC masked the thioT binding site on Aβ fibrils, we examined the thioT fluorescence of Aβ fibrils incubated with purified sCLAC at molar ratios (Aβ/sCLAC) of 1000/1, 250/1, 100/1, 50/1, or 25/1 and confirmed that all samples showed equal levels of fluorescence and contained CLAC-positive fibrils as revealed by immunoelectron microscopy (data not shown), excluding the possibility of masking thioT binding sites in this experimental setting. These results suggested that sCLAC has an ability to inhibit fibrillation of Aβ in vitro.

sCLAC Inhibits Fibrillation of Aβ in Its Elongation Phase—The process of Aβ fibrillation consists of two phases, i.e. nucleation and elongation (Fig. 8A) (4); the nucleation phase represents the conversion of nascent Aβ monomer to an unstructured conformation followed by the formation of fibril seeds. Once fibril seeds are formed, Aβ monomer is rapidly assembled into the seeds or fibrils, eliciting the elongation phase. Thus, the time lag before the formation of fibrils as determined by thioT fluorescence represents the speed of nucleation, whereas the speed of subsequent fibril formation reflects elongation. To investigate whether sCLAC affects either of these two phases of amyloid fibril formation, we incubated Aβ-(1–40) (0.1 mg/ml) alone or together with purified sCLAC, apoE, or α1mG. The use of Aβ-(1–40), which fibrillizes slower than Aβ-(1–42), enabled us to evaluate the effects of coexisting proteins on the nucleation phase, the latter being represented by the time lag before fibril formation. It has been shown that apoE inhibits Aβ fibril formation chiefly in the nucleation phase in vitro (13, 14). Purified sCLAC and apoE inhibited the final extent of fibril formation of Aβ-(1–40), whereas α1mG did not affect this process (Fig. 8B). However, during the initial phase of Aβ fibrillation (0–4 h), the speed of fibrilization of Aβ-(1–40) was similar by coincubation with purified sCLAC or α1mG, whereas apoE inhibited the initial rise in Aβ fibril formation, delaying the start of fibrillation by ~24 h (Fig. 8B). This suggested that sCLAC inhibits Aβ fibril formation in the elongation phase. To further verify this idea, we incubated Aβ-(1–40) (0.1 mg/ml) with prefibrillized/sonication-disrupted Aβ-(1–42) as artificial seeds (molar ratio of Aβ-(1–40)/Aβ-(1–42) seed: 100/1). The addition of a small amount of prefibrillized Aβ-(1–42) strongly promoted the incorporation of Aβ-(1–40) into fibrils, bypassing the nucleation phase (4). In the presence of Aβ-(1–42) seeds, apoE or α1mG no longer affected fibril elongation of Aβ-(1–40), whereas purified sCLAC inhibited the elongation of Aβ fibrils. Taken together, we con-
included that sCLAC has an inhibitory effect on fibrillization of Aβ in its elongation phase.

**DISCUSSION**

In this present study we examined the binding of sCLAC to Aβ in vitro and showed the following. 1) Formation and preservation of the collagenous triple-helix structure of sCLAC is indispensable to its interaction with Aβ; 2) binding of sCLAC with Aβ is mediated by an electrostatic interaction through the positively charged amino acid cluster region in the COL1 domain of sCLAC; 3) fibril formation of Aβ is the prerequisite for its interaction with sCLAC; 4) binding of sCLAC inhibits Aβ fibril formation in the elongation phase. These data provide mechanistic explanation for the selective binding of CLAC to Aβ deposits in AD brains and strongly implicate the inhibitory role of CLAC in β-amyloidosis in AD brains.

We showed that sCLAC is bound to fibrillized Aβ through the positively charged amino acid cluster region in the COL1 domain. Neutralization of the positive charges by proline substitution, suppression of trimer formation by mutation of the coiled-coil domain that is essential to trimer formation, and heat denaturation of sCLAC to disrupt the triple-helix structure altogether abolished binding of CLAC to Aβ, underscoring the importance of the positive charge clusters orderly displayed on the surface of the triple helix. Positively charged amino acid clusters within the collagen regions have been identified as binding sites for a variety of ligands, including Aβ fibrils, in a couple of collagen-related molecules. Type I scavenger receptor class A, known as one of the cell surface receptors for microglial phagocytosis of Aβ fibrils (29), harbors its ligand recognition domain at the collagenous sequence located at the C terminus, in which positively charged amino acids are clustered at the 3rd residues of the collagen repeat sequences. Cells expressing mutant type I scavenger receptor class A substituted at these basic amino acid residues by Ala failed to bind and degrade its major ligand, oxidized low density lipoprotein (30). Complement component C1q, known as one of the amyloid-associated proteins in senile plaques of AD brains, has been shown to interact with Aβ through the positively charged amino acid cluster within the N-terminal collagenous domain of the C1q A chain (31), whereas C1q B or C chains that lack basic amino acid clusters do not bind Aβ. These previous findings are in agreement with our present data and provide further supports to our view that the positively charged amino acid cluster within collagenous domain 1 of CLAC, which is exceptionally rich in positively charged amino acid residues, serves as an optimal binding site for Aβ. This is consistent with our preliminary immunohistochemical data that ectodomain fragments of type XIII or type XXIII collagens do not appear to be associated with senile plaque amyloid.2 Recently Soderberg et al. (32) reported that another positively charged amino acid-rich domain of CLAC, LIKRRLIK, within the non-collagenous domain 2, is the major binding site for Aβ (32); they deleted or replaced these

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2 T. Sakakura, H. Kowa, Y. Matsuura, T. Wakabayashi, T. Hashimoto, and T. Zwatsubo, unpublished data.
amino acid sequences with the homologous region of type XIII collagen that contains smaller number of basic amino acids compared with CLAC and found a reduced Aβ/H9252 binding. This domain seemed to bind to Aβ irrespective of the triple-helix formation of CLAC because synthetic peptides mimicking these sequences were also bound to Aβ. Moreover, they showed that deletion of the basic amino acid cluster within COL1 domain, i.e. KRGKRGRR, which corresponds to the domain we investigated in this present study, did not affect Aβ/H9252 binding. The reason for this apparent discrepancy between these two studies is not clear at present. One possibility would be that a large deletion within the collagen domain, associated with a duplication of glycine residue flanking the deleted portion, might have caused a change in the structure of sCLAC, rendering other positive charge clusters more accessible to Aβ fibrils. In this regard, our strategy not to alter the relative length of each collagenous/non-collagenous domain but neutralize the charges by amino acid substitutions to proline would better preserve the native structure of sCLAC. Another difference is the addition of Myc/His tags in the aforementioned study, which might have caused alterations in structure or charge states. In any event, it remains possible that there are multiple potential Aβ binding sites across the collagenous and non-collagenous domains in CLAC.

We also showed that sCLAC bound fibrillized synthetic Aβ-(1–42) and Aβ-(1–40) but not Aβ-(17–42) despite that the latter formed thioT-positive filaments. This suggests that sCLAC may recognize the N-terminal third of Aβ in its fibrillized form. Indeed, the N-terminal 11 amino acids of Aβ harbor four negatively charged amino acid residues (i.e. DAEFRHDSGYE) that may interact with positive charges on CLAC. Our recent immunohistochemical analysis of postmortem brains from patients with AD or Down’s syndrome showed that CLAC is a relatively early component of senile plaque amyloid coexisting with Aβ42, whereas deposition of Aβ40 occurs chiefly in CLAC-negative senile plaques at a later stage (17). This apparently suggested that CLAC may have different affinities to Aβ42 and Aβ40. However, our preabsorption analysis showed that sCLAC binds to Aβ-(1–42) and Aβ-(1–40) at similar affinities in vitro. It is most plausible that the packing density or structure of Aβ fibrils in Aβ40-positive plaques may be different from those in Aβ40-negative ones in vitro, hampering bind-
ing of CLAC in Aβ40- and thioflavin S-positive SP, whereas in vitro generated Aβ(1–40) and Aβ(1–42) fibrils harbor similar structures in the CLAC binding regions and, thus, exhibit comparable affinities to sCLAC.

We showed by in vitro assays that coinoculation of soluble Aβ with sCLAC inhibited Aβ fibril formation and that this inhibitory effect was chiefly directed to the elongation phase of amyloid fibrils. This process was monitored by thioT fluorescence assay, although a parallel negative-stain electron microscopic analysis confirmed that the decrease in thioT reactivity was not due to masking of thioT binding site by binding of CLAC, because the amount of fibrils generated during incubation was actually reduced. Although the mechanism of this inhibition is still unclear, the effect showed a sharp contrast with that of apoE, which inhibited the seed formation, whereas the addition of prefibrillized seeds overrode this inhibitory effect. It is most plausible to speculate that sCLAC avidly binds to and obstructs the docking sites on Aβ fibrils or protofibrils that serve as seeds for fibril propagation to which monomeric Aβ is successively incorporated, thus inhibiting elongation of

**Fig. 8.** sCLAC inhibits amyloid fibril formation of Aβ in the elongation phase. A, two-phase model of the Aβ fibrillization. In the nucleation phase Aβ monomers slowly change their conformation and form the seeds for fibrils. Once the seeds appear Aβ is rapidly assembled and elongates the fibrils. B, in vitro fibrillization assay of Aβ(1–40). Deseeded Aβ(1–40) (0.25 mg/ml) was incubated in the absence (filled circles) or presence of purified sCLAC (filled squares, molar ratio of Aβ/sCLAC at 100/1), apoE3 (filled triangles, Aβ/apoE3 at 100/1), and α1mG (open circles, Aβ/α1mG at 100/1) for 0, 1, 2, 4, 8, 24, and 48 h, and then thioflavin T fluorescence was quantified. Mean ± S.D. in three independent experiments are shown. C, deseeded Aβ(1–40) (0.1 mg/ml) was incubated together with prefibrillized Aβ(1–42) as a seed (molar ratio of Aβ(1–40)/seed Aβ(1–42): 100/1) in the absence (filled circles) or presence of a purified sCLAC (filled squares; Aβ/sCLAC: 100/1), α1mG (open circles), or apoE3 (filled triangles) for 0, 1, 2, 4, 8, 24, or 48 h. Deseeded Aβ(1–40) alone was incubated as a control (filled diamonds). The mean ± S.D. of three independent experiments is shown.
Aβ fibrils. Further in vitro studies to examine the binding of sCLAC to Aβ polymers in various states, i.e., oligomers, protofibrils, and fibrils, will be needed.

Our present in vitro data strongly suggest that CLAC may serve as an inhibitory factor for Aβ fibrillization that slows down the deposition of β-amyloid in AD brains. However, the possibility that CLAC binding may have multiple downstream effects that variously influence Aβ deposition should be reserved. For example, collagens harboring triple-helix structure are known to be resistant against conventional proteinases, suggesting that CLAC deposited in senile plaques may confer amyloid deposits resistance to proteolytic degradation or microglial phagocytosis. Indeed, we have shown that Aβ immunoreactivities in CLAC-positive senile plaques are more resistant to proteinase K digestion compared with those in CLAC-negative ones (33). Considering the discrepancies in the effects of apoE on Aβ fibrillization and deposition that were previously documented in vitro (inhibitory (13, 14)) and in vivo (promoting (12)), however, further in vivo studies, especially cross-breeding experiments of transgenic mice overexpressing AβPP and CLAC-P, will be mandatory. This will provide important clues to the elucidation of the pathological roles of CLAC in the deposition of β-amyloid and AD as well as of its therapeutic potentials.

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CLAC Binds to Amyloid β Peptides through the Positively Charged Amino Acid Cluster within the Collagenous Domain 1 and Inhibits Formation of Amyloid Fibrils
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Page 8602, Footnote 2: Dr. Iwatsubo's last name was misspelled in the footnote at the bottom of the right column.