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# Structural Requirement of Fibrogenic Laminin-Derived Peptide A119 (LSNIDYILIKAS) for Amyloid-like Fibril Formation and Cellular Activity

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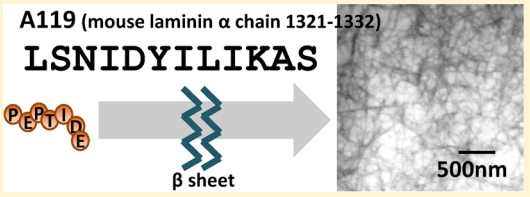
Supporting Information

**ABSTRACT:** A119 peptide (LSNIDYILIKAS), derived from the mouse laminin $\alpha$1 chain sequence (residues 1321−1332), promotes cell attachment, neurite outgrowth, and amyloid-like fibril formation. In this study, we evaluated the structural requirements of A119 for biological activities and amyloid-like fibril formation. The attachment of the cell to A119 was inhibited by heparin, and using syndecan- and glypican-overexpressed cells, it was determined that A119 specifically binds to syndecans. We also evaluated the critical residues for A119 activities using a set of alanine-substituted peptides. Cell attachment activity was significantly reduced in the Leu[1]-, Ser[2]-, Asn[3]-, Ile[4]-, Ile[7]-, Ile[9]-, and Lys[10]-substituted alanine peptides. Residues Ile[4], Ile[7], Ile[9], and Lys[10] were important for neurite outgrowth activity. Congo red staining and electron microscopic examination revealed that the Ile[4], Ile[7], Ile[9], and Ser[12] residues of A119 were required for amyloid-like fibril formation. These data suggest that the Ile residues are critical for the amyloid-like fibril formation, cell attachment, and neurite outgrowth activity of A119. Furthermore, an enantiomer of A119 showed similar amyloid-like fibril formation and increased levels of cell attachment and FAK signal transduction. These findings shed light on the mechanism of amyloid-like fibril formation and demonstrate a relationship between the ability to form amyloid-like fibrils and cell behavior.




It is known that the process of amyloid fibril formation, including amyloid $\beta$ (A$\beta$) and islet amyloid polypeptide, is generally associated with diseases such as Alzheimer's disease, type II diabetes, and prion diseases.[1,2] However, many proteins form amyloid-like fibrils but are not associated with amyloid-related diseases.[3−5] Amyloid fibrils are elongated, insoluble structures 7−10 nm in diameter found in extracellular plaques.[6] Structural studies have shown that mature amyloid fibrils involve the alignment of short peptide segments, usually between 6 and 12 residues in length, from many protein monomers. Together, these aligned polypeptide stretches form the characteristic core structure of the amyloid fibril, the cross $\beta$ sheet, in which $\beta$ strands run perpendicular to the main axis of the fibril. Amyloid fibrils possess a characteristic UV absorbance spectrum at the peak of 540 nm with Congo red and a characteristic X-ray diffraction pattern.[7,8] Thus, a specific pattern of molecular interactions, rather than nonspecific hydrophobic interactions, leads to ordered structures. Nevertheless, common structural elements responsible for such organized structures have not been identified, and the mechanism of amyloid fibril formation is unclear.

Previously, we screened the synthetic peptide library derived from the laminin-111 sequence and identified five amyloidgenic peptides, A119 (LSNIDYILIKAS, mouse laminin $\alpha$1 chain, residues 1321−1332), A208 (AASIKVAVSADR, mouse laminin $\alpha$1 chain, residues 2097−2108), AG97 (SAKVDAIGLEIV, mouse laminin $\alpha$1 chain, residues 2942−2953), B133 (DISTKYFQMSLE, mouse laminin $\beta$1 chain, residues 1367−1378), and B160 (VILQQSAADIAR, mouse laminin $\beta$1 chain, residues 1607−1618).[9] Laminins, major components of basement membranes, regulate multiple biological functions, including cell adhesion, migration, differentiation, neurite outgrowth, wound healing, angiogenesis, and tumor progression.[10−12] Laminins make up a family of heterotrimeric glycoproteins that consist of $\alpha$, $\beta$, and $\gamma$ chains. So far, five $\alpha$, three $\beta$, and three $\gamma$ chains have been identified, and at least 16 laminin isoforms that are formed by various combinations of these chains have been reported.[13]

Some of these fibril-forming peptides have been studied with respect to their structural requirements for fibril formation and biological activity. Laminin-derived peptide A208, containing the Ile-Lys-Val-Ala-Val (IKVAV) sequence, which interacts with a 110 kDa membrane-associated laminin-binding protein,[14] forms amyloid-like fibrils.[15] We found previously that the IKVAV sequence was required for fibril formation and for biological activities, including cell attachment and neurite outgrowth.[15] Additionally, an enantiomer of peptide A208 (a208) promotes similar biological activities.[15] We have also reported that B133 promotes syndecan- and integrin $\alpha2\beta1$-mediated cell attachment, neurite outgrowth, and amyloid fibril formation.[16] The N-terminal Asp-deleted peptide B133a (SITKYFQMSLE) maintained $\alpha2\beta1$-mediated cell attachment activity, although neurite outgrowth activity and amyloid-like fibril formation were eliminated.[16] On the other hand, the C-terminal Glu-deleted peptide B133g (DSITKYFQMSL)

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exhibited syndecan-mediated cell attachment, neurite outgrowth, and amyloid-like fibril formation.[17] Furthermore, an alanine scan of B133g suggested that the critical residues for amyloid-like fibril formation and syndecan-mediated cell attachment were $Asp^1$ and $Ile^3$, and $Asp^1$ for neurite outgrowth activity.[17]

Here, we focus on the A119 peptide, which exhibits amyloid-like fibril formation, heparin-dependent cell attachment activity, and neurite outgrowth activity.[9] The receptor and the structure−activity relationship between amyloid-like fibril formation and the biological activities of the A119 peptide have not been investigated. Our approach was to determine the cellular receptors for the A119 peptide and to investigate the essential residues for fibril formation and cell behavior using alanine-substituted peptides. Understanding the amyloid fibril formation mechanism of peptide A119 is important for understanding general amyloid-like fibril formation.

## MATERIALS AND METHODS

**Synthetic Peptides.** All peptides were prepared by the *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) solid phase synthesis with a C-terminal amide. The respective amino acids were condensed using diisopropylcarbodiimide-*N*-hydroxybenzotriazole on a 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin [Rink amide resin (Novabiochem, San Diego, CA)]. Resulting protected peptide resins were deprotected and cleaved from the resin using a trifluoroacetic acid (TFA)/thioanisole/*m*-cresol/ethanedithiol/$H_2O$ mixture (80:5:5:5:5, v/v) at room temperature for 3 h. Crude peptides were precipitated and washed with diethyl ether and then purified by high-performance liquid chromatography (HPLC) using a Mightysil RP-18 column (Kanto Chemical Co., Inc., Tokyo, Japan) with a $H_2O$/acetonitrile gradient containing 0.1% TFA. The purity and identity of the synthetic peptides were confirmed by HPLC and by electrospray ionization mass spectroscopy. Mass spectroscopy was performed at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences. Peptides were dissolved in water at 1 mM, kept at 4 °C for 1 week, and then used for the assay.

**Congo Red Binding Analysis.** A 100 μM stock solution of Congo red was prepared in phosphate-buffered saline (PBS) and 10% ethanol. Ethanol was added to prevent Congo red micelle formation. This Congo red stock solution was filtered three times using a 0.45 Micron Nylon Membrane (Iwaki Co. Ltd., Tokyo, Japan). The peptide solutions in water (100 μL, dissolved at a final concentration of 0.1 mM) and the Congo red stock solution (100 μL) were mixed with 800 μL of PBS (1.25×) and incubated in disposable cuvettes for 24 h at room temperature in the dark. Absorption spectra were measured from 300 to 700 nm using a UV-1700 UV−vis spectrophotometer (Shimadzu Co. Ltd., Kyoto, Japan).

**Electron Microscopy.** A peptide solution (1 mM) was diluted from 1:0 to 1:4 in water and applied to a grid mesh with carbon-coated Formvar film [a 0.5% ethylene dichloride solution of polyvinyl formal (Nisshin EM Co. Ltd., Tokyo, Japan)]. The specimen was then negatively stained with a 1.5% aqueous solution of uranyl acetate and observed using a JEM-1011 (JEOL Ltd., Tokyo, Japan) electron microscope at an acceleration voltage of 80 kV.

**Cells and Culture.** Human neonatal dermal fibroblasts (HDFs) (AGC Techno Glass Co., Ltd., Chiba, Japan) were maintained in Dulbecco's modified Eagle's medium [DMEM (Invitrogen, Carlsbad, CA)] containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Rat pheochromocytoma, PC12 cells[18] were maintained in DMEM containing 7.5% horse serum, 7.5% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The series of human ARH77 B-lymphoid cell lines, which are transfected with heparan sulfate proteoglycans (syndecan-1, -2, and -4 and glypican-1),[19,20] were grown in suspension in RPMI 1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 5% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin. All cells were maintained at 37 °C in a humidified, 5% $CO_2$ and 95% air atmosphere.

**Cell Attachment Assay Using Peptide-Coated Plates.** The attachment of cells to peptide-coated plates was assayed in 96-well plates (Nunc, Inc., Naperville, IL). Plates were coated with various amounts of peptide in water and dried overnight. The peptide-coated wells were blocked with 1% bovine serum albumin [BSA (Sigma, St. Louis, MO)] in DMEM (150 μL) for 1 h and then washed twice with DMEM containing 0.1% BSA. Cells were detached by trypsin and EDTA, resuspended in DMEM containing 0.1% BSA, and added to each well (20000 cells/100 μL), and the wells were incubated at 37 °C for 1 h in 5% $CO_2$. The attached cells were stained with a 0.2% crystal violet aqueous solution in 20% methanol for 10 min. After being washed with water, the attached cells were photographed using a BZ-8000 microscope (Keyence, Osaka, Japan). Images were captured and analyzed using BZ-analyzer (Keyence). The attached cells in three randomly selected fields were counted. All assays were run in triplicate with each experiment repeated at least three times.

**Neurite Outgrowth.** For the neurite outgrowth assay, 96-well plates (Nunc) were coated with various amounts of peptides and dried overnight. PC12 cells were primed with 100 ng/mL nerve growth factor [NGF (Roche Diagnostics GmbH, Mannheim, Germany)] for 24 h prior to the assay. The PC12 cells were then released from the dish by agitation, collected by centrifugation, allowed to recover in culture medium for 30 min at 37 °C in 5% $CO_2$, and then washed twice with a DMEM/F12 mixture (Wako). After being washed, cells were resuspended in a DMEM/F12 mixture containing 100 μg/mL transferrin (Sigma), 20 nM progesterone (Sigma), 30 nM $Na_2SeO_3$ (Wako), 5 μg/mL insulin (Invitrogen), and 100 ng/mL NGF. The cells (5000 cells per 100 μL per well) were added to 96-well plates. After incubation at 37 °C for 24 h in 5% $CO_2$, the cells were fixed with 20% formalin and then stained with 0.2% crystal violet. After being washed with water, the attached cells were photographed using a BZ-8000 microscope (Keyence). Images were captured and analyzed using BZ-analyzer (Keyence). In each well, 100 cells were viewed, and the percentage of active cells, which had neurites that extended a length twice the cell diameter or longer, was determined.

**Immunoblotting of FAK Signaling.** After HDFs were incubated on the various peptide-coated plates (20000 cells/well) for 90 min, the cells were lysed with 50 μL of 4× Laemmli sample buffer. Then, Western blotting was performed using anti-phospho-focal adhesion kinase (FAK) $Tyr^{397}$ or anti-FAK polyclonal antibody (Cell Signaling Technology, Danvers, MA). The relative phosphorylation of phospho-FAK $Tyr^{397}$ was assessed using ImageJ version 1.46 (http://imagej.nih.gov/ij).

**Statistical Analysis.** Results were expressed as ± the standard deviation (SD). Comparison of mean values was


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performed using one-way analysis of variance, and a homoscedastic $t$ test. $P < 0.01$ indicated statistical significance.

## RESULTS

**Effect of EDTA and Heparin on the Attachment of Cells to A119.** Previously, we reported that A119 has amyloid-like fibril formation activity and can promote cell attachment and neurite outgrowth.[9] First, we examined the effect of EDTA and heparin on the attachment of cells to A119 to better define the cell surface receptor (Figure 1). AG73 (RKRLQVQLSIRT,

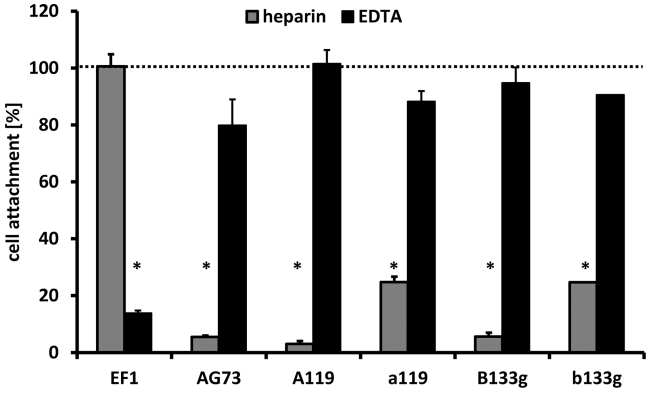


**Figure 1.** Effect of EDTA and heparin on the attachment of HDF to A119. Plates (96 wells) were coated with peptides (A119, a119, B133g, and b133g at a density of 2 nmol/well, EF1 at 0.25 nmol/well, and AG73 at 1 nmol/well). HDF suspensions were preincubated with either 5 mM EDTA or 10 $\mu$g/mL heparin. Then, the cells were added and incubated. After a 30 min incubation, the number of attached cells was assessed by crystal violet staining. Data are shown as means ± SD of triplicate results. *$P < 0.01$ vs none.

mouse laminin $\alpha$1 chain, residues 2719−273),[21] which binds to syndecan, EF1 (DYATLQLQEGRLHFMFDLG, mouse laminin $\alpha$1 chain, residues 2747−2765),[22] which binds to integrin $\alpha2\beta1$, and B133g (DISTKYFQMSL, mouse laminin $\beta$1 chain, residues 1367−1377),[17] which forms amyloid-like fibrils and binds to syndecan, were used as controls. As expected, the attachment of cells to EF1 was inhibited by only EDTA and that to AG73 and B133g was inhibited by only heparin (Figure 1). Only heparin significantly inhibited the attachment of cells to A119, suggesting that A119 promotes heparan sulfate proteoglycan-mediated cell attachment.

**Attachment of A119 to Heparan Sulfate Proteoglycan-Overexpressing Lymphoid Cells.** The A119 peptide promoted heparin-dependent cell attachment. We examined which heparan sulfate proteoglycans recognized the A119 peptide using syndecan-1-, -2-, and -4- and glypican-1-overexpressing ARH77 B-lymphoid cells (synd-1, synd-2, synd-4, and glp-1, respectively). ARH77 cells express very low levels of cell surface heparan sulfate proteoglycans. AG73, EF1, and B133g were used as controls. AG73 and B133g have syndecan-specific binding,[17,23] while EF1 promotes integrin $\alpha2\beta1$-mediated cell attachment.[22] AG73 and B133g attached only to the syndecan-overexpressing cells, and EF1 did not promote attachment to the syndecan- and glypican-1-overexpressing cells (Figure 2). The A119 peptide promoted attachment to the syndecan-overexpressing cells but did not promote attachment to the glypican-1-overexpressing cells (Figure 2). The results suggest that amyloid-like fibril-forming peptide A119 promotes syndecan-mediated cell attachment.

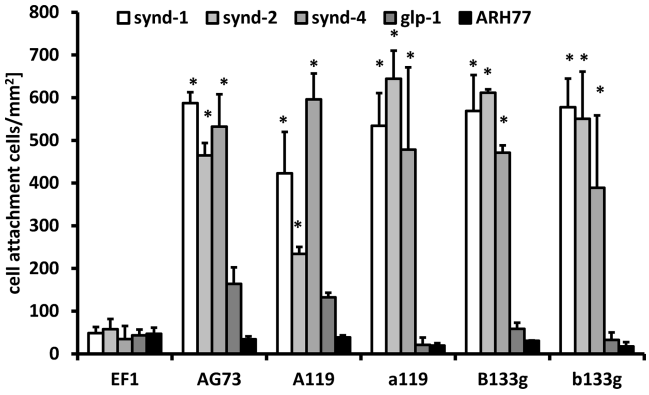


**Figure 2.** Cell attachment activity of the amyloidogenic peptides with various heparan sulfate proteoglycan-overexpressing cell lines. Heparan sulfate proteoglycan-overexpressing cell lines with syndecan-1 (synd-1), syndecan-2 (synd-2), syndecan-4 (synd-4), and glypican-1 (glp-1) and controls without these receptors (ARH77) were used. Each peptide was coated on a 96-well plate. After each plate had been blocked with 1% BSA in DMEM, 20000 cells were added to the well and incubated for 1 h. After the cells had been washed, the number of attached cells was assessed by crystal violet staining. Data are shown as means ± SD of triplicate results. *$P < 0.01$ vs ARH77.

**Effect of Amyloidogenic Peptides on FAK Signaling.** FAK is an important component of focal contacts, and FAK in focal contacts undergoes autophosphorylation of its major phosphorylation site, tyrosine 397. $Tyr^{397}$ phosphorylation of FAK is critical for focal contacts and actin cytoskeleton formation. EF1, an integrin binding peptide, induced intense phosphorylation, as shown previously.[24] B133g also promoted the $Tyr^{397}$ phosphorylation of FAK, but AG73 did not exhibit this activity. A119 did not induce the $Tyr^{397}$ phosphorylation of FAK (Figure 3). These results suggest that A119 does not influence cellular function via integrins.

**Effect of Alanine-Substituted A119 Peptides on the Congo Red Absorption Spectrum.** Next, we evaluated the amino acid sequence requirements of A119 for amyloid-like fibril formation. We prepared alanine-substituted A119 peptides and analyzed the absorption spectrum of the peptide solutions with Congo red (Table 1 and Figure 4). Congo red binds to amyloid fibrils and promotes an absorption peak shift from 490 to 540 nm.[25] Previously, we reported that A119 exhibited Congo red binding ability.[9] The A119I7A and A119I9A peptides, in which $Ile^7$ and $Ile^9$ were substituted with Ala, respectively, lost the transition of UV absorption spectra with Congo red staining. The $Ile^4$-substituted peptide (A119I4A) significantly reduced the absorption at 540 nm (Figure 4). The $Leu^1$-, $Ser^2$-, $Asn^3$-, and $Ser^{12}$-substituted Ala peptides reduced the absorption at 540 nm, but that reduction was smaller than that of the Ile-substituted Ala peptides. Ala substitution of the other residues did not affect the absorption peak of Congo red. These data suggest that the $Ile^4$, $Ile^7$, and $Ile^9$ residues in A119 are critical for amyloid-like fibril formation.

**Analysis of Amyloid-like Fibrils by Electron Microscopy.** We examined the fibril formation activity of the alanine-substituted A119 peptides using negative staining electron microscopy (Figure 5). A119 exhibited typical amyloid-like fibrils. A119I7A and A119I9A did not form fibrils. A119I4A formed fibrils, but they were shorter than those of A119. $Ser^{12}$-substituted peptide A119S12A formed globular aggregates. Other alanine-substituted peptides maintained amyloid-like fibril activity. These results are comparable with those of the


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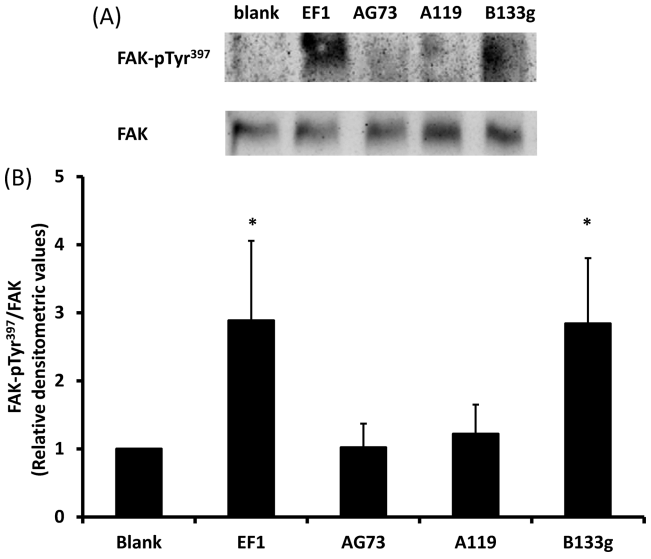


**Figure 3.** FAK Tyr[397] phosphorylation on amyloidogenic peptides. (A) FAK Tyr[397] phosphorylation and FAK of HDFs on 1 nmol/well peptides. The HDFs were incubated on the various peptide-coated plates for 90 min, lysed by addition of 80 μL of SDS sample buffer, and assessed by Western blotting. (B) Quantification of panel A. The relative phosphorylation of FAK Tyr[397] was assessed using ImageJ version 1.46. Data are shown as means ± SD of triplicate results. *$P$ < 0.01 vs blank.

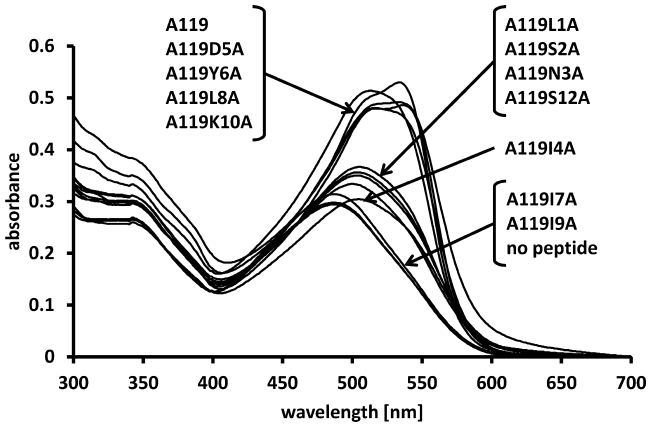


**Figure 4.** Congo red binding analysis of A119 and its alanine-substituted peptides. Peptides dissolved in water (100 μL, 1 mM) and a Congo red solution (100 μL, 100 μM in PBS) were mixed with 800 μL of 1.25× PBS and incubated for 24 h at room temperature. Absorption spectra from 300 to 700 nm were recorded.

Congo red binding analysis and strongly suggest that Ile[7] and Ile[9] are critical and Ser[12] is important for amyloid-like fibril formation.

**Cell Attachment Activity of Alanine-Substituted A119 Peptides.** We evaluated the cell attachment activities of alanine-substituted A119 peptides using HDFs. The HDF attachment activity was significantly decreased when Leu[1], Ser[2], Ile[7], and Ile[9] were substituted with Ala. Further, the peptides in which Gln[3], Ile[4], Lys[10], and Ser[12] were substituted with Ala lost cell attachment activity (Figure 6). The other alanine substitutions did not affect cell attachment activity. These results suggest that Leu[1], Ser[2], Ile[7], and Ile[9] are important and that Gln[3], Ile[4], Lys[10], and Ser[12] are critical for cell attachment activity.

**Neurite Outgrowth Activity of Alanine-Substituted A119 Peptides.** We also evaluated the neurite outgrowth activity of the A119 alanine-substituted peptides. A119 promoted neurite outgrowth with PC12 cells, as described previously.[9] Neurite outgrowth was not stimulated on the Ile[4]-, Ile[7]-, Ile[9]-, and Lys[10]-substituted alanine peptides (Figure 7). The remainder of the alanine-substituted peptides showed neurite outgrowth activities like that of A119. Morphological differences were not observed among the active peptides (Figure 7). These results suggest that Ile[4], Ile[7], Ile[9], and Lys[10] are critical for the neurite outgrowth activity of the A119 peptide.

**Biological Activities of the A119 Enantiomer.** The A208 peptide, an IKVAV sequence-containing peptide, requires amyloid-like fibril formation for biological activity, including cell attachment and neurite outgrowth.[15,26] Previously, an enantiomer of A208 (a208) was also shown to promote

**Table 1. Biological Activities of A119 and Its Alanine-Substituted Derivatives**

| peptide | sequence | Congo red staining[a] | amyloid-like fibril formation | cell attachment[b] (HDFs) | neurite outgrowth (PC12) |
|---|---|---|---|---|---|
| A119 | LSNIDYILIKAS | +++ | + | ++ | + |
| A119L1A | **A**SNIDYILIKAS | ++ | + | + | + |
| A119S2A | L**A**NIDYILIKAS | ++ | + | + | + |
| A119N3A | LS**A**IDYILIKAS | ++ | + | -- | + |
| A119I4A | LSN**A**DYILIKAS | + | -- | -- | -- |
| A119D5A | LSNI**A**YILIKAS | +++ | + | ++ | + |
| A119Y6A | LSNID**A**ILIKAS | +++ | + | ++ | + |
| A119I7A | LSNIDY**A**LIKAS | -- | -- | + | -- |
| A119L8A | LSNIDYI**A**IKAS | +++ | + | ++ | + |
| A119I9A | LSNIDYIL**A**KAS | -- | -- | + | -- |
| A119K10A | LSNIDYILI**A**AS | +++ | + | -- | -- |
| A119S12A | LSNIDYILIKA**A** | ++ | -- | -- | + |

[a]Peptides were incubated with a Congo red solution, and the absorption spectrum at 540 nm was evaluated on the following subjective scale: +++, strong spectrum compared to that of A119; ++, spectrum comparable to that of A119; +, weak spectrum compared to that of A119; −, no shift in the absorption peak. [b]Cell attachment activities were scored on the following subjective scale: ++, activity comparable to that on A119; +, weak activity compared with that on A119; −, no activity.


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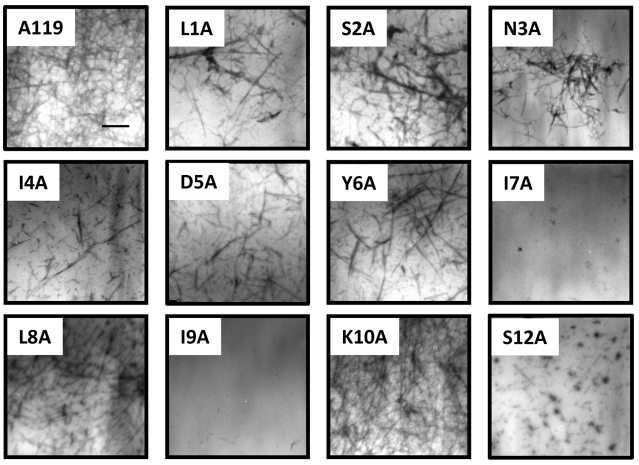


**Figure 5.** Electron micrographs of amyloid-like fibril-formed peptides. Peptide solutions (0.25−1 mM) were applied to a grid mesh with carbon-coated Formvar film. Each specimen was then negatively stained with a 1.5% aqueous solution of uranyl acetate and observed using an electron microscope. The scale bar represents 500 nm.

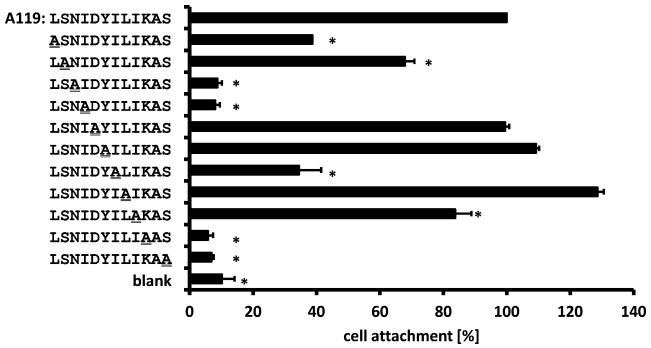


**Figure 6.** Cell attachment activity of A119 and its alanine-substituted peptides. Plates (96 wells) were coated with 10 nmol of A119 per well and its alanine-substituted peptides and examined for cell attachment activity using HDFs; 20000 cells were added to each well for 1 h. After the cells had been washed, the number of attached cells was assessed by crystal violet staining. A119 was used as a relative control for activity. Data are shown as means ± SD of triplicate results. *$P < 0.01$ vs A119.

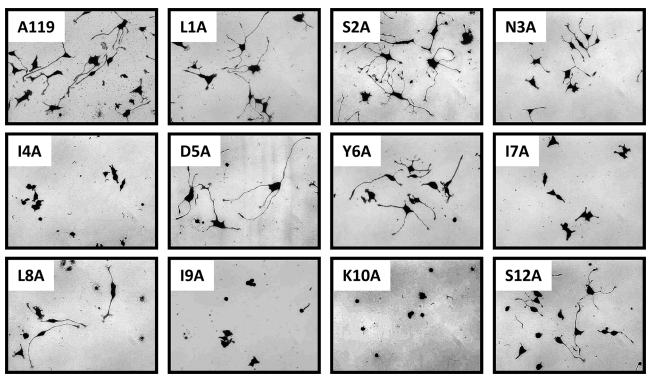


**Figure 7.** Neurite outgrowth of PC12 cells on A119 and its alanine-substituted peptides. Peptides (10 nmol/well) were coated on 96-well plates. PC12 cells (5000 cells/well) were cultured on various peptides for 24 h and then stained with crystal violet.

amyloid-like fibril formation, cell attachment, and neurite outgrowth as well as A208.[15] We prepared enantiomers of A119 and B133g (a119 and b133g, respectively) and evaluated their biological activities. The a119 and b133g peptides formed amyloid-like fibrils by Congo red staining (Table 2). The a119 peptide increased HDF attachment activity compared with that of A119, while b133g decreased HDF attachment activity compared with that of B133g (Table 2). The HDF attachment was inhibited by only heparin (Figure 1). The a119 peptide induced $Tyr^{397}$ phosphorylation of FAK, although A119 did not (Table 2). The FAK signal transduction on B133g was not observed on b133g (Table 2). The a119 peptide showed neurite outgrowth activities similar to that of A119, but b133g lost activity (Table 2). To identify the cellular receptors, we examined cell attachment activity using proteoglycan-over-expressing ARH77 cells (synd-1, synd-2, synd-4, and glp-1). Both a119 and b133g exhibited cell attachment activity with respect to synd-1, synd-2, and synd-4, but not glp-1 (Figure 2). These results suggest that the a119 and b133g peptides promote syndecan-mediated cell attachment.

## ■ DISCUSSION

Amyloid fibril formation is generally related to diseases, such as Alzheimer's disease, Parkinson's disease, type II diabetes, prion diseases, and systemic polyneuropathies. Recently, although many studies have reported on the mechanism of amyloidogenesis,[27,28] studies of the peptide sequences involved in amyloid fibril formation are limited. Electron microscopy showed that peptide A119 forms amyloid-like fibrils (Figure 5). Amyloid fibrils are known to be self-cohesive and insoluble, and the structure is built up by stacking $\beta$ strands to form a cross $\beta$ structure. The fibrils are not dependent on the hydrophobicity of the peptide, but on specific interactions between peptides. For example, the A$\beta$(1−42) peptide in its natural structure forms self-assembled insoluble fibrils, while its *O*-acyl isopeptide structure at $Gly^{25}$-$Ser^{26}$ remains soluble and does not form fibrils.[29] Amyloidogenesis of a peptide depends on the primary structure, and an amino acid residue replacement or deletion can "switch" between amyloid-forming and non-amyloid-forming peptides.[29]

Previously, we reported five new amyloidogenic peptides derived from the laminin-111 sequence, A119, A208, AG97, B133, and B160.[9] In this study, we prepared A119 Ala-substituted peptides and assayed amyloid-like fibril formation and biological activities (Table 1). Our results suggested that $Ile^4$, $Ile^7$, $Ile^9$, and $Ser^{12}$ play a critical role in amyloid-like fibril formation. The A119I7A and -I9A peptides could not form amyloid-like fibrils (Figure 5). To investigate the secondary structure of A119 and its Ala-substituted peptides, we used circular dichroism (CD) spectra (see the Supporting Information). The CD spectrum of A119 showed typical $\beta$ strand structure; however, the CD spectra of A119I7A and -I9A showed typical $\alpha$ helical structure. Therefore, the $Ile^7$ and $Ile^9$ residues are critical for the process of $\beta$ strand formation. The A119I4A and -S12A peptides formed short fibrils and globular aggregates (Figure 5). The CD spectrum of these peptides indicated that they formed mixed secondary structure ($\alpha$ helix and $\beta$ strand). It is expected that amyloid-like fibril formation of these peptides was imperfect because the $Ile^4$ and $Ser^{12}$ residues are important for either intramolecular $\beta$ strand formation or the process of shifting from a $\beta$ sheet to a cross $\beta$ sheet. We wonder whether the globular aggregates of the A119S12A peptide resulted in hydrophobic interaction. Substitution of $Ser^{12}$ with Ala makes it impossible to form $\beta$ strand and increases hydrophobicity, which leads to nonspecifically


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**Table 2. Biological Activities of Enantiomers of Amyloidogenic Peptides**

| peptide | sequence[a] | Congo red staining[b] | cell attachment (HDFs)[c] | neurite outgrowth (PC12)[d] | cellular receptor | phosphorylation of $Tyr^{397}$ FAK |
|---|---|---|---|---|---|---|
| A208[e] | AASIKVAVSADR | + | ++ | ++ | 110 kDa protein | --[f] |
| a208[e] | aasikvavsadr | + | ++ | + | (unknown) | (not tested) |
| A119 | LSNIDYILIKAS | ++ | + | + | syndecan | -- |
| a119 | lsnidyilikas | ++ | ++ | ++ | syndecan | + |
| B133g | DISTKYFQMSL | ++ | ++ | ++ | syndecan | + |
| b133g | distkyfqmsl | ++ | + | + | syndecan | -- |

[a]D-Configuration amino acids are indicated by the lowercase letters. [b]Peptides were incubated with a Congo red solution, and the absorption spectrum at 540 nm was evaluated on the following subjective scale: ++, spectrum comparable to that of 119; +, weak spectrum compared to that of A119. [c]Cell attachment activities were scored on the following subjective scale: ++, activity comparable to that on A119; +, weak activity compared with that on A119. [d]Neurite outgrowth activities were scored on the following subjective scale: ++, strong activity compared to that of A119; +, spectrum comparable to that of A119; −, no activity. [e]The biological activities of enantiomers of A208 were previously described.[15] [f]Unpublished data.

hydrophobic aggregation. Previously, we examined amyloid-like fibril-forming peptide B133 derived from the laminin $\beta$1 chain sequence using its truncated peptides and Ala-substituted peptides.[17] We reported that the $Asp^1$ and $Ile^3$ residues of the B133 sequence are critical for amyloid-like fibril formation.[17] CD spectra of the Ala-substituted B133g peptides indicated that $Asp^1$-substituted B133g01 preserved a $\beta$ strand structure of B133g but $Ile^3$-substituted B133g03 did not.[17] These data suggest that the Ile residues are "commonly" important for amyloid-like fibril formation through intramolecular $\beta$ strand formation.

We also clarified that the $Leu^1$, $Ser^2$, $Asp^3$, $Ile^4$, $Ile^7$, $Ile^9$, $Lys^{10}$, and $Ser^{12}$ residues are important for the cell attachment activity of A119 (Figure 6). These results suggested that there are two factors for cell attachment; one is fibril formation activity, and the other is a positive charge. Because the $Ile^4$, $Ile^7$, $Ile^9$, and $Ser^{12}$ residues are critical for amyloid fibril formation, fibril formation was thought to promote cell attachment. Furthermore, the A119 sequence includes the Lys residue, which has positive charge, and this positive charge may interact with the negative charge of syndecans. The Lys residue is exposed outside of the amyloid-like fibril, and A119 likely interacts with syndecans.

The $Leu^1$, $Ser^2$, $Asp^3$, and $Ser^{12}$ residues are important for cell attachment activity but not necessary for neurite outgrowth activity. The $Ile^4$, $Ile^7$, $Ile^9$, and $Lys^{10}$ residues were critical for neurite outgrowth activity and signal transduction in these cells (Figure 7). Recently, it was reported that PC12 cell neurite outgrowth was promoted by RhoE, which activated rac and cdc42 through syndecans.[30] Because A119 did not promote phosphorylation of FAK, A119 might promote neurite outgrowth by stimulating intracellular signaling through rac1 and cdc42. Although amyloid-like fibril formation promotes cell attachment of A119 through syndecans, neurite outgrowth by A119 may depend on the primary structure.

The enantiomer of the A208 peptide, a208, forms amyloid-like fibrils and has biological activity similar to that of the parent molecule (Table 2).[15,26] We examined amyloid-like fibril formation and biological activities using enantiomers of A119 and B133g (a119 and b133g, respectively). Both enantiomers of A119 (A119 and a119) showed syndecan-mediated HDF attachment and neurite outgrowth activities (Figures 1 and 2 and Table 2). However, $Tyr^{397}$ phosphorylation of FAK was observed with the a119 peptide, but not with the A119 peptide (Table 2). On the other hand, although both enantiomers of B133g (B133g and b133g) exhibited syndecan-mediated HDF attachment, the neurite outgrowth activity and FAK signal transduction were lost with the b133g peptide (Table 2). A119 and B133g have similar properties, e.g., amyloid-like fibril formation, neurite outgrowth activity, and syndecan-mediated cell attachment. We hypothesized that amyloid-like fibril formation was accomplished in two steps: (1) formation of a $\beta$ sheet structure by the peptide via intramolecular distribution and (2) intermolecular directional migration. Because both a119 and b133g formed amyloid-like fibrils, it is suggested that the amyloid-like fibril was formed in a manner independent of chirality.[15] Syndecans have negatively charged sugar chains, which might recognize the surface of amyloid-like fibrils. On the basis of the data showing that attachment of HDF to the $Lys^{10}$-substituted peptide was lost (Table 1 and Figure 6), we conclude that the chirality had little influence on the cell attachment activities, and that the syndecans recognized positive charges on amyloid-like fibrils. However, for neurite outgrowth activity and FAK signal transduction, the chirality might be very important. Syndecan-4 is known to promote $Tyr^{397}$ phosphorylation of FAK through activation of $\beta$1 integrin.[31,32] In this study, A119, B133g, and their enantiomers did not exhibit syndecan-4-specific binding (Figure 2). The A119 peptide might bind syndecans nonspecifically or weakly bind to syndecan-4; therefore, phosphorylation of FAK was not observed. The enantiomeric isomerization gave A119 a new binding property while B133g lost the activity. On the basis of our hypothesis relative to the biological activities, A119 and B133g were comparable to b133g and a119, respectively. In other words, the "native" side of A119 amyloid-like fibrils was equal to the "opposite" side of B133g amyloid-like fibrils.

Taking those results into consideration, we concluded that A119 forms amyloid-like fibrils mainly via the Ile residues and exhibits cell attachment activity based on specific fibril structure specifically via an interaction with heparan sulfate, regardless of their chirality. Additionally, the Ile and Lys residues in the A119 peptide sequence stimulate intracellular signaling that promotes neutrite outgrowth and $Tyr^{397}$ phosphorylation of FAK depending on the amino acid residues and chirality.

Elucidation of the amyloid-like fibril formation mechanism will affect the future medical treatment of amyloid-related diseases. Alzheimer's disease is incurable, and there is an unmet need for new medications and new approaches to treatment. In


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Japan, the only treatment is donepezil, which is based on dopamine restoration and is used to increase the level of acetylcholine in the brain of Alzheimer's disease patients.[33] Although there is delayed progression of the demented state, this treatment is not a cure. At present, many researchers are actively searching for inhibitors of amyloid fibril formation.[34−36] Understanding amyloid fibril formation mechanisms will lead to the development of new treatments that target amyloid fibrils. The results of this study provide additional information about amyloid-like fibril formation mechanisms.

## ■ ASSOCIATED CONTENT

### Supporting Information

CD spectra of A119 and its alanine-substituted peptides mentioned in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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