

Ile-Lys-Val-Ala-Val (IKVAV)-containing laminin α 1 chain peptides form amyloid-like fibrils

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Abstract The Ile-Lys-Val-Ala-Val (IKVAV) sequence derived from laminin-1 promotes cell adhesion, neurite outgrowth, and tumor growth and metastasis. Here, we examined amyloid formation of an IKVAV-containing peptide (LAM-L: AASIK-VAVSADR, mouse laminin α 1 chain 2097–2108). The LAM-L peptide was stained with Congo red and exhibited fibrils in electron microscopy with a characteristic cross- β X-ray diffraction pattern. Further, infrared spectra of LAM-L suggested a β -sheet structure. These results indicate that LAM-L forms amyloid-like fibrils. We also examined amyloid-like fibril formation of LAM-L analogs. The neurite outgrowth activity of the LAM-L analogs was closely related to their amyloid-like fibril formation.

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1. Introduction

Laminins, the major components of basement membranes, comprise a family of large heterotrimeric glycoproteins [1]. So far, five α , three β , and three γ chains have been identified, and at least 15 isoforms are formed by various combinations of each subunit [2–4]. Laminin-1, consisting of three chains designated α 1, β 1, and γ 1, has diverse biological activities including promotion of cell adhesion, migration, neurite outgrowth, tumor metastasis, and angiogenesis [1]. Laminin-1 is over-expressed in both Alzheimer's and Down's brains [5], especially in the frontal cortex of Alzheimer's disease [6]. These findings suggest that laminin α 1 chain has a potential to be involved in the Alzheimer's disease.

Several active sequences of laminin-1 have been identified using synthetic peptide approaches [7–11]. An Ile-Lys-Val-Ala-Val (IKVAV) sequence located on the C-terminal end of the long arm of the α 1 chain promotes cell adhesion, neurite outgrowth, angiogenesis, collagenase IV production, and

tumor growth [12–16]. A 110 kDa membrane-associated laminin-binding protein from brain binds to the IKVAV site and functions to promote neurite outgrowth on IKVAV [17]. This membrane protein has been identified as β -amyloid precursor protein [18].

Recently, several peptide segments of proteins were shown to self-assemble and to form amyloid-like fibrils [19,20]. Amyloid-like fibrils bind to Congo red [21] and appear as uniform unbranched fibrils [22]. Congo red binding analysis is a quick method for determination of amyloid-like fibril formation [21–24] and is widely used for determination of amyloid-like structures. The mechanism of the Congo red interaction is not well understood [25,26]. Amyloid-like fibrils play a critical role in various diseases, including Alzheimer's, the transmissible spongiform encephalopathies, type II diabetes mellitus, and systemic polyneuropathies [27–29]. Laminin and its fragments interact with β -amyloid proteins, inhibit fibril formation, and are present in Alzheimer's plaques [30].

Using Congo red staining, electron microscopy, X-ray diffraction, and infrared (IR) spectroscopy, we describe amyloid-like fibril formation of the IKVAV-containing 12-mer peptide LAM-L, derived from the laminin α 1 chain. Structural requirements of the IKVAV-containing peptides for amyloid-like fibril formation are also evaluated.

2. Materials and methods

2.1. Synthesis of peptides

All peptides were synthesized manually by 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase methods and purified by reverse-phase high performance liquid chromatography (HPLC) as described previously [16,31]. Purity of the peptides was confirmed by analytical HPLC. Identity of the peptides was confirmed by analysis in a mass spectrometer.

2.2. Congo red binding analysis

Peptide solution in phosphate-buffered saline (PBS) (0.1–2.5 mg/ml) and Congo red solution (100 μ M in PBS) were mixed and incubated in disposable cuvettes for various times at room temperature. Absorption spectra were measured from 300 to 700 nm using a U-2000A UV-Vis Spectrophotometer (Hitachi Co. Ltd., Tokyo, Japan).

2.3. Congo red staining and polarized light microscopy

LAM-L was dissolved in PBS at a concentration of 5 mg/ml, and the solution was pipetted onto a glass slide. After drying overnight, the precipitate was stained with a 1% aqueous solution of Congo red

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Abbreviations: IR, infrared

for 1 h. After rinsing with pure acetone, the samples were dehydrated with 95% ethanol and 100% ethanol and then cleared with xylene. The specimens were mounted with a resin (malinol, Muto pure chemicals, Tokyo, Japan) and observed in a microscope (AX80, Olympus, Tokyo, Japan) either under bright field illumination or between crossed polars.

2.4. Electron microscopy

A grid mesh was coated with a thin Formvar (polyvinyl formal, Ohken, Tokyo, Japan) film and then stabilized by evaporating a carbon layer. LAM-L gel in PBS (5 mg/ml) was applied on the grid mesh and then stained with a 5% aqueous solution of uranyl acetate and observed using a JEM-1200EX (JEOL, Tokyo, Japan) electron microscope at an acceleration voltage of 80 kV.

2.5. X-ray diffraction

LAM-L, dissolved in H₂O (5 mg/ml), was packed into a siliconized capillary glass tube (ϕ 10 μ m, Verpackung ges, Berlin, Germany), and dried for 3 days in a wetting box at room temperature. The precipitate of LAM-L was produced at the top of the capillary glass tube.

X-ray diffraction patterns were obtained at room temperature using CuK α (λ = 1.5418 Å) radiation from a M18XXHF (50 kV, 90 mA, MAC Science Co., Ltd., Yokohama, Japan) with double focusing mirrors. Data were collected on a DIP-R300 diffractometer (MAC Science). The specimen-to-film distance was set at 150 mm and the exposure time was 30 min. X-ray diffraction patterns were displayed and measured using the xdisp (MAC Science).

2.6. Fourier transform IR spectrometer

Aqueous LAM-L solution (50 μ l, 5 mg/ml LAM-L in H₂O) was applied onto a Teflon[®] plate, dried for 2 weeks in a wetting box at room temperature, and then stripped from the plate. The IR absorption spectra for the dried LAM-L were measured by KBr methods using a Fourier transform IR spectrometer (RT-210, Horiba Co.,

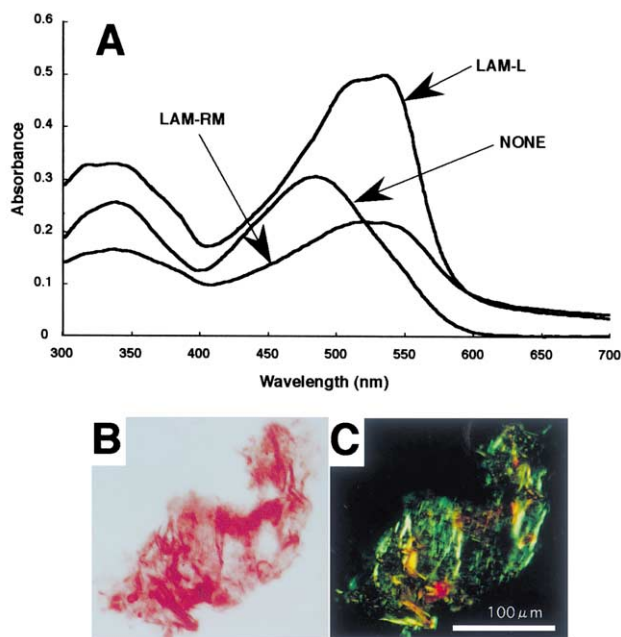


Fig. 1. Absorption spectra of Congo red solution in the presence of peptides and photomicrographs of the LAM-L peptide stained with Congo red. A: Congo red solution (100 μ l, 100 μ M in PBS) and peptide solution (500 μ l, 2 mg/ml in PBS) were mixed in PBS (400 μ l) at room temperature for 24 h and UV spectra were measured from 300 to 700 nm. B and C: LAM-L in PBS (5 mg/ml) was pipetted onto a glass slide. After drying overnight, the precipitate was stained with 1% aqueous solution of Congo red for 1 h. After rinsing with pure acetone, the samples were dehydrated with 95% ethanol and 100% ethanol and then cleared with xylene. The specimens were mounted with a resin and observed in a microscope either under bright field illumination (A) or between crossed polars (B).

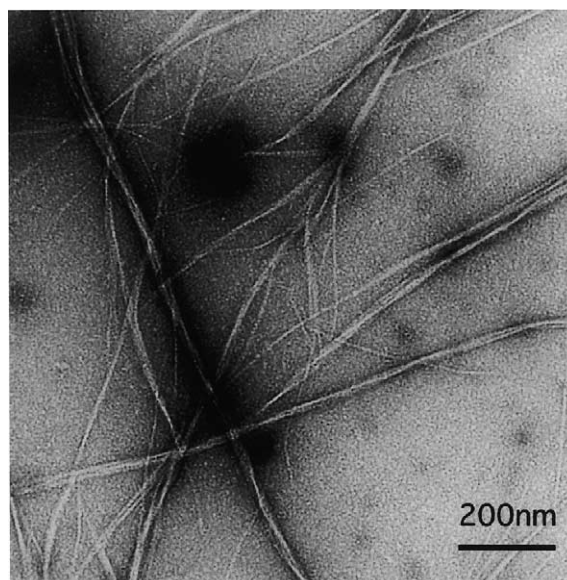


Fig. 2. Electron micrograph of amyloid-like fibrils. LAM-L (1 mg) was dissolved in PBS (200 μ l), and the resulting gel was kept at 4°C for 1 week. The amyloid-like fibrils were stained with a 5% aqueous solution of uranyl acetate and observed using an electron microscope.

Ltd., Kyoto, Japan). The IR spectrum was measured with a resolution of 4 cm^{-1} .

3. Results

3.1. Congo red staining analysis

The ability of a laminin α 1 chain active peptide LAM-L (AASIKVAVSADR) and the scrambled peptide LAM-RM to form amyloid-like fibrils was tested using Congo red [32,33]. The absorption spectrum of the Congo red solution showed a peak at 486 nm as described previously [34] (Fig. 1A). When the Congo red solution was incubated with LAM-L for 24 h, two larger peaks at 512 and 536 nm appeared (Fig. 1A). The absorbance at 512 and 536 nm specifically increases when Congo red binds to amyloid-like fibrils, such as amyloid β -peptides [34–36]. LAM-RM had a small effect on the absorption spectrum of Congo red, but the absorbance at 512 and 536 nm was not significantly increased. These results suggest that the LAM-L peptide binds to Congo red in a sequence-specific manner.

Next, LAM-L solution (5 mg/ml) was pipetted onto a slide glass, dried, and stained with Congo red. When the sample was observed under a polarizing microscope, the peptide precipitate exhibited birefringence, going from red to green (Fig. 1B and C). These results further suggest that the LAM-L peptide forms amyloid-like structure and significantly binds to Congo red.

3.2. Formation of amyloid-like fibrils

We next examined amyloid-like fibrils formed by self-assembly of LAM-L. The LAM-L peptide was dissolved in PBS and analyzed by negative staining electron microscopy. The LAM-L solution (5 mg/ml in PBS) formed a gel which had the appearance of amyloid-like fibrils (Fig. 2). This result strongly suggests that the LAM-L peptide self-assembles and forms amyloid-like fibrils.

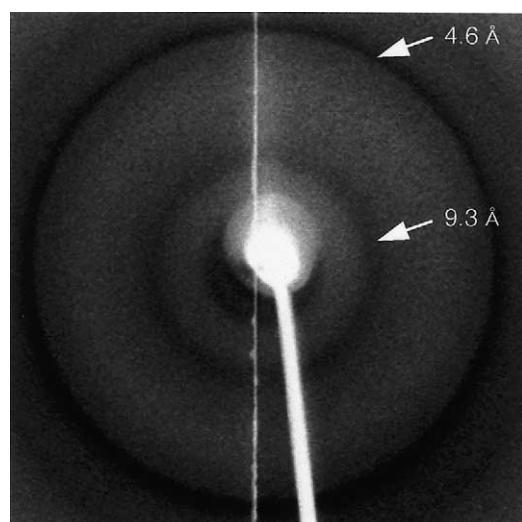


Fig. 3. X-ray diffraction pattern of LAM-L. Two major reflections of the amyloid fibrils (4.6 and 9.3 Å) are marked by arrows. Due to the poor alignment of fibrils, those reflections appear as rings.

The X-ray diffraction pattern of the LAM-L gel showed two major reflections that appear as rings due to the poor alignment of the fibrils (Fig. 3). A dominant sharp reflection was observed at a position corresponding to 4.6 Å and a weaker one was observed at 9.3 Å (Fig. 3). These reflections are characteristic of the cross- β conformation described for many amyloid fibrils [19,22]. In well-oriented amyloid fibril samples, the 4.6 Å reflection is a characteristic distance and corresponds to the interstrand spacing in the cross- β conformation, while the 9.3 Å one is equatorial and corresponds to the intersheet spacing [19,22]. The X-ray data suggest that the LAM-L peptide forms a cross- β conformation.

The IR spectrum of the LAM-L, determined using the KBr method, showed one prominent band at 1635 cm^{-1} in the amide I region (Fig. 4). An amide group in the β -sheet conformation gives rise to highly diagnostic bands between approximately 1620 and 1640 cm^{-1} in the amide I region [37,38]. The IR spectra of the LAM-L suggest that the peptide possesses a β -sheet conformation in the dried state. In addition, a β -sheet conformation of LAM-L in dried state was also confirmed by the circular dichroism (CD) spectrum (data not

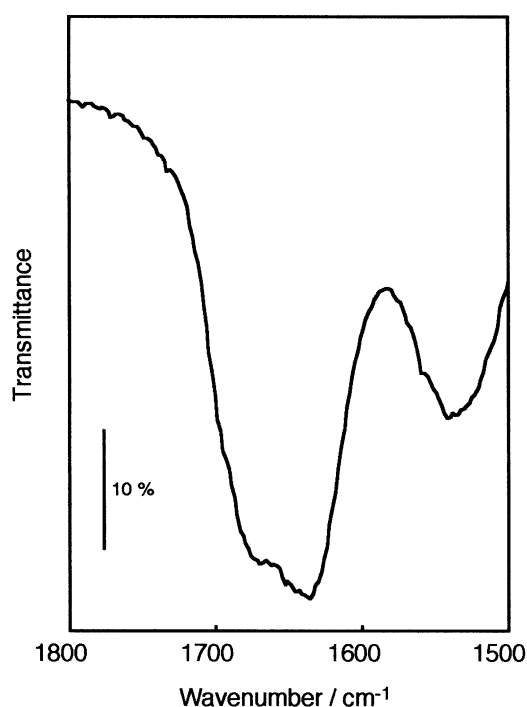


Fig. 4. Fourier transform IR spectrum (KBr method) of LAM-L. Aqueous LAM-L solution was applied onto a Teflon® plate, dried for 2 weeks in a wetting box, and then stripped from the plate.

shown). A similar CD spectrum was previously obtained with LAM-L in methanol [16].

3.3. Congo red staining analysis of LAM-L analogs

Amyloid-like fibrils formed from LAM-L analogs (Table 1) were analyzed by Congo red analysis (Fig. 5). The analog peptides were designed based on 12-mers containing the IKVAV sequence and having cell adhesion and neurite outgrowth activity [31]. Congo red solution was mixed with peptides and then the absorbance at 536 nm was measured at various times (Fig. 5). An all-D-configuration peptide, LAM-D, a mirror image compound of LAM-L, shifted the absorption spectrum of the Congo red solution similar to that observed with LAM-L (Fig. 5), while LAM-LR and LAM-

Table 1
Effect of IKVAV analog peptides on PC12 cells attachment and neurite outgrowth and Congo red binding

Peptide	Sequence ^a	Cell attachment ^b	Neurite outgrowth ^b	Congo red assay ^c
LAM-L	AASIKVAVSADR	++	++	++
LAM-D	<u>AASIKVAVSADR</u>	++	+	++
LAM-LR	RDASVAVKISAA	—	N.D.	—
LAM-DR	<u>RDASVAVKISAA</u>	—	N.D.	—
LAM-RM	AASVVIKASADR	—	N.D.	—
LAM-KR	AASIRVAVSADR	++	+	+
LAM-KE	AASIEVAVSADR	—	N.D.	—
LAM-IL	AASLKVAVSADR	+	—	—
LAM-IX	AASXKVAVSADR	++	+	++
LAM-IV	AASVKVAVSADR	++	—	—
LAM-IA	AASAKVAVSADR	—	N.D.	—
AG73	RKRLQVQLSIRT	++	++	—
AG73T	LQQRRSVLRTKI	—	—	—

^aD-configuration amino acids are indicated by the underline. Substituted amino acids are indicated in bold.

^bCell attachment and neurite outgrowth activities of the peptides were described previously [16,31,43].

^cThe Congo red assay was evaluated on the following subjective scale: ++, activity comparable to that of LAM-L; +, activity weaker than that of LAM-L; —, inactive. N.D. denotes not done.

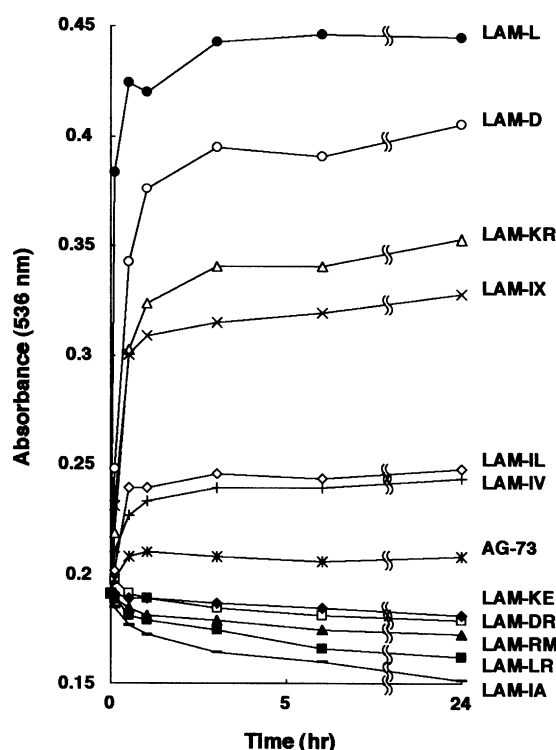


Fig. 5. Congo red staining of LAM-L analogs (time course). Congo red solution (100 μ l, 100 μ M in PBS) and peptide solution (100 μ l, 1 mg/ml in PBS) were mixed in PBS (800 μ l) and absorption at 536 nm was measured at various times.

DR, all-L and all-D reverse-sequence peptides that are biologically inactive, did not affect the absorption spectrum of the Congo red solution. These results suggest that the effect on the absorption spectrum of Congo red correlates with the biological function.

LAM-L analog peptides with single amino acid substitutions were also tested for their effect on the absorption spectrum of the Congo red solution (Fig. 5). Analogs in which the lysine residue in LAM-L was replaced with either arginine or glutamic acid (LAM-KR and LAM-KE, respectively) were used (Table 1). Analogs in which the isoleucine residue in LAM-L was substituted with either leucine, norleucine, valine, or alanine (LAM-IL, -IX, -IV, and -IA, respectively) were also tested (Table 1). LAM-KR and LAM-IX increased UV absorbance at 536 nm, but all other analogs were inactive (Fig. 5). These results suggest that the isoleucine and lysine positions in the IKVAV sequence are important for amyloid-like formation as well as for the biological activity.

4. Discussion

Here, we have demonstrated that biologically active laminin peptides containing the IKVAV sequence can form amyloid-like fibrils. Amyloid structure, self-assembly of β -sheet domains resulting in the formation of an ordered fibrillar structure, is a common feature of various medical disorders. These include infectious prion diseases, e.g. bovine spongiform encephalopathy or Creutzfeldt–Jacob disease, amyloidoses, such as Alzheimer's disease or type II diabetes, and Huntington's disease [27–29,39]. Characteristic for these diseases is that amyloidogenic precursor proteins are converted into insoluble

pathogenic protein fibrils (amyloids). We focused on the IKVAV sequence-containing peptide LAM-L and examined its amyloid-like fibril formation. When the peptides were examined using Congo red, LAM-L strongly shifted the absorption spectrum of Congo red. Similar shifts to the long wavelength have been observed for amyloid-like structures, such as with amyloid β -peptides [33–36]. When the LAM-L gel was stained with Congo red, the peptide precipitate exhibited birefringence, going from red to green. Additionally, microfibrils were observed when LAM-L was examined by electron microscopy. Further, X-ray diffraction and IR analysis indicated that the fibrils possessed a β -sheet structure. These results suggest that the LAM-L peptide promotes the formation of an amyloid-like structure. Moreover, a structure activity study using Congo red staining and the cell adhesion and neurite outgrowth activity with the LAM-L peptide and its analog peptides suggested that the amyloid-like formation of the IKVAV peptides correlated with biological activity. These results suggest that the IKVAV-containing laminin α 1 chain peptides form amyloid-like fibrils and play a critical role in biological activity.

The all-D peptide (LAM-D), previously shown to have cell attachment and neurite outgrowth activities [16,31], promoted amyloid-like fibril formation as well as that of LAM-L (Table 1), while the reverse-sequence all-L and all-D peptides (LAM-LR and LAM-DR, respectively) were not biologically active and did not promote amyloid-like fibril formation. These results indicate that both all-L and all-D IKVAV peptides can self-assemble and form amyloid-like fibrils, however, the all-L and all-D VAVKI-containing peptides (a reverse sequence of IKVAV peptide) cannot self-assemble. Additional structure activity studies using LAM-L analogs with single amino acid substitutions suggested that amyloid-like fibril formation and biological activity were correlated (Table 1). Thus, the structural requirements of the IKVAV peptide for interaction with its cell surface receptor or binding proteins are specified by the primary structure.

Laminin interacts with various amyloid proteins and may be involved in amyloid formation [18,30,40,41]. The IKVAV-containing peptides interact with the 110 kDa amyloid precursor protein [18]. Laminin binds to β -amyloid protein and inhibits fibril formation, suggesting that the binding peptide segment may have potential for therapeutic use in inhibition of β -amyloid protein fibrillogenesis [30]. The binding site of β -amyloid protein is likely localized in the C-terminal region of the laminin α 1 chain [30]. The IKVAV sequence may be involved in the laminin– β -amyloid protein interaction.

Recently, we identified 72 active peptides from 673 overlapping peptides covering the laminin-1 molecule using the peptide-coated plate and peptide-conjugated Sepharose bead assays [8–11]. 28 peptides promoted cell attachment in both assays. 49 peptides showed cell attachment activity only in the peptide-coated plate assay, and 51 peptides were active only in the peptide-conjugated Sepharose bead assay. Some of the peptides that showed activity in both assays were found to interact with integrins and syndecans [8–11,42]. In this study, we used the most active peptide of the laminin α 1 chain AG73 (LQVQLSIR) as a control. AG73 promoted cell attachment in both peptide-coated plate and peptide-conjugated Sepharose bead assays, while it did not affect the absorption spectrum of the Congo red solution (Table 1 and Fig. 5). LAM-L was active only in the plate assay. It is possible that the peptides

that were only active in the peptide-coated plate coat assay may form an amyloid-like formation similar to that of the IKVAV peptide, but this has not yet been tested.

Laminin may have a potential role in Alzheimer's disease [5,6]. Laminin and its fragments also have been suggested to be involved in the amyloid formation [5,6,30]. In this study, we described the IKVAV peptide formed amyloid-like fibrils. These findings suggest that the amyloid-like fibril formation of the IKVAV region may play a critical role in biological function.

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