In Vitro Amyloidogenic Peptides of Galectin-7
POSSIBLE MECHANISM OF AMYLOIDOGENESIS OF PRIMARY LOCALIZED CUTANEOUS AMYLOIDOSIS

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Background: Characterization of amyloid precursor protein and the mechanism of amyloidogenesis in primary localized cutaneous amyloidosis have not been elucidated previously.

Results: Galectin-7 fragments containing β-strand peptides are highly amyloidogenic in vitro.

Conclusion: Galectin-7 is an amyloid precursor protein in primary localized cutaneous amyloidosis.

Significance: We have proposed the possible mechanism of amyloid deposition in primary localized cutaneous amyloidosis.

Primary localized cutaneous amyloidosis (PLCA) is a skin lesion characterized by chronic pruritic papules (lichen amyloidosus) or macules (macular amyloidosis) and histologically characterized by the deposition of amyloid in the superficial dermis (1). PLCA is relatively common in South America and Southeast Asia, and most cases are the acquired type with unknown pathogenesis. The amyloid precursor protein of PLCA has been hypothesized to be cytokeratins based on the immunohistochemical reactivity of amyloid deposits with cytokeratin antibodies (2, 3). The immunohistochemical positivity with cytokeratins in the lesional skin, however, does not always indicate that cytokeratins are amyloidogenic precursor proteins. Because cytokeratins are major intracellular proteins of keratinocytes, they have been excluded from the candidates for amyloid precursor protein (4). In addition, it has been demonstrated that KIF and its proteolytic fragments do not react with thioflavin T (ThT) (4). Furthermore, the secondary structure of cytokeratin consists of α-helical, coiled-coil rod domains (5), whereas more than 20 amyloidogenic proteins that can bind Congo red or ThT share common cross-β sheet structures, which is defined as β-fibril- lization (6). Recently, we identified galectin-7 (Gal-7), actin, and cytokeratins as well as serum amyloid P component (SAP) and apolipoprotein E (Apo E) as the major components of amyloid deposits of PLCA in the water soluble fraction recovered from lesional skin, and we found that all of these proteins were present in the amyloid deposits of PLCA according to immunohistochemical studies (2, 7–9). SAP and Apo E are universal non-fibrillar constituents of amyloid deposits and will therefore be excluded from the candidates for amyloid precursor protein. The next questions are which protein (Gal-7, actin, and cytokeratins) is directly related to amyloidogenesis and how the protein aggregates to form amyloid fibrils.

The initial event of amyloid formation in PLCA is thought to be related to keratino- cyte apoptosis because 1) TUNEL-posi-
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tive keratinocytes are commonly detected in the epidermis overlying the amyloid-laden dermis (10), and 2) amyloid deposits are restricted to the papillary dermis just beneath the thinned epidermis, suggesting that a hyperproliferative thickened epidermis undergoes extensive apoptotic cell death (filamentous degeneration) (11) and is then substituted by amyloid materials via an unknown mechanism. Apoptosis of keratinocytes, which is induced by UVB irradiation, reactive oxygen species (ROS), drugs or chemical reagents accompanies the activation of certain proteolytic machinery (12). Three major apoptosis-related enzymes of keratinocytes have been reported, including caspases, cathepsin D (cath D), and trypsin-like enzymes, although their final target molecules and their exact roles in apoptosis are not fully known. Nine caspases are expressed in normal human keratinocytes; of these, caspases 3, 8, and 9 are activated in UVB-induced apoptosis (13); in particular, caspase 3 appears to be important because its activity is enhanced by apoptosis-induced Gal-7 (14). Cath D, a major lysosomal, acidic protease of the epidermis, induces apoptosis via directly priming caspase-8 activation (15, 16). Keratinocytes potentially synthesize multiple forms of trypsin-like serine pro teaseas including trypsinogen 4, 5, and kallikrein-related pepti des (KLKs) (17, 18). A trypsin serine protease secreted by cul tured keratinocytes potentially induces keratinocyte apoptosis, which is blocked by soybean trypsin inhibitor, but the precise mechanism is currently unknown (19).

The general mechanism of amyloid fibril formation is not fully understood. A nucleation-dependent polymerization model is proposed for the general mechanism of amyloid fibril formation in several types of amyloidosis, such as β2-microglobulin in dialysis amyloidosis and amyloid-β (Aβ) in Alzheimer disease. Their amyloidogenic models consist of two fundamental phases, nucleation (seed formation), and extension (deposition). Seed formation requires the assembly of monomeric protein into a template, representing the rate-limiting step in amyloid fibril formation. Once the seed has been formed, growth of the template by the deposition of additional monomeric protein becomes thermodynamically favorable, resulting in rapid extension of amyloid fibrils (20, 21).

Galectins constitute a large family of β-galactoside-binding lectins. At least 14 mammalian galectins that share structural similarities in their carbohydrate recognition domains have been reported. Despite the lack of signal peptide, galectins can be secreted by an endoplasmic reticulum (ER)-Golgi-independent pathway. They can be found both intracellularly (cytoplasmic and/or nuclear) and extracellularly as well as, sometimes, in association with the plasma membrane, although they do not contain a transmembrane domain. From these variable subcellular locations, galectins have been implicated in a wide range of cellular processes, including cell-cell and cell-matrix interactions, extracellular matrix remodelling, cell cycle, cancer biology, intracellular trafficking, and apoptosis. Some galectins are pro-apoptotic, whereas others are anti-apoptotic; some galactins induce apoptosis by binding to cell surface glycoproteins, whereas others regulate apoptosis through interacting with intracellular proteins (22, 23). Gal-7 is a prototype galectin, with a molecular weight of 14 kDa (136 amino acid residues), that is expressed only in stratified epithelia (24). Its structure consists of β-sandwich with the packing of two β-sheets (25). Gal-7 is associated with multiple keratinocyte functions including cell migration and apoptosis. Gal-7-deficient mice display reduced reepithelialization potential compared with wild-type littermates (26). Gal-7 expression is markedly induced during UVB-induced apoptotic processes of epidermal keratinocytes, paralleling p53 stabilization (27); the keratinocytes overexpressing Gal-7 undergo apoptosis, indicating that Gal-7 is a pro-apoptotic protein (14, 28).

In the present study, we 1) determine the amyloidogenic potentials of the candidate proteins, Gal-7, actin, and cytokeratins; 2) determine which part of Gal-7 is involved in amyloidogenesis; and 3) show whether degraded fragments of Gal-7 produced by keratinocyte apoptosis-related proteases can form amyloid fibrils.

EXPERIMENTAL PROCEDURES

Proteins and Synthetic Peptides—Recombinant human Gal-7 expressed from cDNA clone (29) was purchased from R&D systems, Inc. (Minneapolis, MN). The purity of the sample was more than 97% as determined by SDS-PAGE. The structure of human galectin-7 consists of paired β-sheets, F1-F5 and S1-S6a fragments (see Fig. 2, A and B) (25). The β-strand-containing fragments of Gal-7, Gal S1-F2 (NH2-PHKSSLPEGIRPGTVLIRGLVPP-COOH), Gal S3-S5 (NH2-SRFHVNLLCGEEQGSDAALHFNPRDLTSEVVFNSEQ-COOH), Gal S3-S4 (NH2-SRFHVNLLCGEEQGSDAALHFNPRDLTSEVVFNSEQ-COOH), Gal S4-S5 (NH2-DAAHFNPRDLTSEVVFNSEQ-COOH), Gal S5-S6a (NH2-SEVVFNSEQGSGWGREERGP-COOH), Gal S6b-S6a (NH2-GSWGREERGP-COOH), Gal F3-F5 (NH2-QGFPEVLIIASDDGFKAATRVDAQYQHFRHR-COOH), and Gal S2-F1 (NH2-RLVEVGQDDLQDSRIF-COOH) were synthesized with the solid phase method (Filgen, Inc., Bioscience Department, Nagoya, Aichi, Japan). These peptides were designed to contain one extra amino acid residue at both the amino and C-terminal ends of the β-strand domains (see Fig. 2B). Analogs of the Gal S3-S5 (amino acid number Ser31-Gln67) fragment lacking 1–4 residues at the N- or C-terminal end, including Phe33-Lys65, Ser31-Lys65, Arg32-Glu66, Phe33-Ser64, Val55-Lys65, His54-Lys65, Phe53-Asn65, and Phe33-Gln67, and those of Gal S2-F1 (amino acid number Arg120-Phe136) lacking 1–2 residues at the N or C end, including Leu121-Ar134, Leu121-Ile135, Arg120-Ile135, Leu121-Phe136, were also synthesized (See Fig. 2B). The purities of the fragments were more than 85% based on the supplier’s HPLC data.

Human non-muscle actin (β and γ) purified from platelet was purchased from Cytoskelton, Inc. (Denver, CO), and the purity was >99%. Four species of synthetic peptides of β-actin, which contain an extra amino acid residue at both the N- and C-terminal ends of β-strand domains (30), Leu-Pro38 (NH2-LVVDNQSGMCKAGFADDPRAVPSVGRP-COOH), Val55-Lys68 (NH2-IVGPRPHQGMVQGMQKDSYGDAEAQSKRGILTLK-COOH), Gly150-Leu178 (NH2-TGIVMDSDGVTHTVIPYE1GYALPHAILRLD-COOH), and Lys238-Ile250 (NH2-EEKSYELPDGQV1TIG-COOH) were prepared as described above, and the purities were more than 90%.

Insoluble cyto keratinins were extracted from cultured human keratinocytes (HaCaT cells) in phosphate-buffered saline (PBS) containing 0.6 M KCl, 1% Triton X-100, and 0.2 mg/ml DNase I.
(31). The precipitate was dissolved in an 8 M urea/0.1 M β-ME at room temperature for 4 h and then centrifuged at 10,000 × g for 30 min. The supernatant was dialyzed four times against PBS; then, the resulting aggregates, which largely comprise cytokeratins (referred as insoluble cytokeratins), were redissolved in 8 M urea/0.1 M β-ME solution and reaggregated by dialysis against PBS. The procedure was repeated four times (4). Salt soluble cytokeratins were extracted from the insoluble cytokeratin aggregates using the buffer (10 mM Tris-HCl, pH 7.5) to give the same final concentration of Me2SO. Proteins or peptides were easily dissolved in water, but some were difficult. In some experiments, we prepared a stock solution of the peptides (f(Phe33-Lys65)/Phe33-Lys65 monomer, or heterogeneous combination of f(Phe33-Lys65)/Phe33-Lys65 monomer, or heterogeneous combination of f(Ser31-Gln67)/Gal-7 (Met1-Phe136) monomer.

Modulation of Amyloidogenesis of Gal-7 Fragments—Amyloidogenic Gal-7 peptides, Ser31-Gln67 and Phe33-Lys65, were incubated at their amyloidogenic dose (200 μM) in 50 mM citrate buffer, pH 2.0, containing 100 mM NaCl in the presence of Gal-7 fragments (Gal S1-F2, Gal S5-S6a, and Gal F3-F5) (200 μM each), and its β-strand domains (Leu8-Pro32, Val35-Lys68, Gly150-Leu178, and Lys238-Ile250) (200 μM each), and cytokertatin digests (20–200 μM) prepared by 3 h trypsin treatment (see below) for 0, 3, 6, 10, and 18 h.

Amyloidogenic Gal-7 peptides, Arg120-Phe136, Leu121-Arg134, and Leu121-Phe136, were incubated at their poorly amyloidogenic dose (100 μM) in 50 mM citrate buffer, pH 2.0 containing 100 mM NaCl in the presence of Gal-7 fragments (Gal S1-F2, Gal F3-F5, and Gal S5-S6a) (100 μM each), and cytokertatin digests (100 μM) for 10, 24, 48, 72, and 96 h.

Proteolytic Digestion of Gal-7, Actin, and Cytokeratins—Recombinant Gal-7 (200 μg), non-muscle actin (200 μg) and insoluble cytokeratin aggregates isolated from cultured keratinocytes (300 μg) were digested for 1, 3, and 8 h with trypsin (sequence grade, Promega, WI) (10 μg/ml) in 50 mM Tris-HCl buffer, pH 8.0, at room temperature; cathepsin D (Sigma-Aldrich Inc., MO) (3 μg/ml) in 50 mM acetate buffer pH 4.0 at 37°C; or caspase 3 (Sigma-Aldrich Inc.) (5 μg/ml) in 50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, and 0.1% CHAPS at 37°C. The reaction was stopped by the addition of pepstatin A (1 μg/ml) (Wako Pure Chemical Industries Ltd.) in the case of cathepsin D digestion or by the addition of acid buffer (1 mM citrate buffer, pH 2.0) in the trypsin and caspase 3 digestions. An aliquot was dissolved in SDS sample buffer (0.1 M Tris-HCl, pH 7.0 containing 10% SDS) and then subjected to
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SDS-PAGE to determine the degree of the degradation of the substrates. The reaction mixtures in 100 mM citrate buffer, pH 2.0, containing 100 mM NaCl were further incubated at 37 °C for 6, 24, 48, and 72 h to determine the amyloidogenic potentials of the degradation products with the ThT method and electron microscopy.

RESULTS

Fluorescence Spectra of ThT in the Presence of Amyloid Fibrils Extracted from PLCA—Water-soluble amyloid fibrils (fPLCA) (30 μg/ml) were extracted from lesional skin of PLCA and showed a novel fluorescence at 485 nm with an excitation maximum at 435 nm. Amyloid fibrils (30 μg/ml) produced a novel fluorescence at 485 nm with an excitation maximum at 435 nm. B, fluorescence spectra of galectin-7 (50 μM) was monitored before (dotted line) and after 3 h of incubation (solid line) at pH 2.0 (left panel) and pH 7.0 (right panel). C and D, effect of pH or the incubation time on the ThT fluorescence. Galectin-7, actin, or soluble keratin at 50 μM was incubated for 3 h at various pH ranges or for 3, 6, 18, and 24 h at pH 2.0 or 4.0. Values are normalized to those of 500 μM Gal-7 at pH 2.0 after 3 h incubation (see Fig. 3, A and B). E, electron micrographs of the structures appeared after the incubation of galectin-7 at pH 2.0 for 3 h. The bar indicates 200 nm.

Fluorescence of ThT in the Presence of Galectin-7 (Gal-7), Actin, and Cytokeratin Molecules—The emission spectra of the Gal-7, actin, and cytokeratin molecules at the dose of 50 μM each were determined with an excitation spectrum at 435 nm. Gal-7 showed a novel peak at 485 nm at pH 2.0 after 3 h, but there was no fluorescence peak at pH 7.0 (Fig. 1B). Actin and cytokeratins showed no fluorescence at both pH 2.0 and 7.0 (not shown).

The effects of pH on ThT fluorescence in the presence of Gal-7, actin, and soluble keratin (50 μM each) were examined. ThT fluorescence of Gal-7 was pH-dependent with a maximal fluorescence after 3 h at pH 2.0, while actin and soluble keratin showed no increase in ThT fluorescence in any pH ranges (Fig. 1C).

The effects of the incubation time on the ThT fluorescence in the presence of Gal-7 (50 μM) at pH 2.0 and 4.0, as well as actin and cytokeratins at pH 2.0, were examined. Gal-7 (50 μM) gave a maximal fluorescence after 3 h at both pH 2.0 and 4.0 with a

FIGURE 1. Detection of amyloidogenic potentials of galectin-7, actin, and cytokeratins by ThT method and electron microscopic observation. A, fluorescence spectra of amyloid fibrils (fPLCA) that were isolated from the lesional skin of primary localized cutaneous amyloidosis (PLCA). Amyloid fibrils (30 μg/ml) produced a novel fluorescence at 485 nm with an excitation maximum at 435 nm. B, fluorescence spectra of galectin-7 (50 μM) was monitored before (dotted line) and after 3 h of incubation (solid line) at pH 2.0 (left panel) and pH 7.0 (right panel). C and D, effect of pH or the incubation time on the ThT fluorescence. Galectin-7, actin, or soluble keratin at 50 μM was incubated for 3 h at various pH ranges or for 3, 6, 18, and 24 h at pH 2.0 or 4.0. Values are normalized to those of 500 μM Gal-7 at pH 2.0 after 3 h incubation (see Fig. 3, A and B). E, electron micrographs of the structures appeared after the incubation of galectin-7 at pH 2.0 for 3 h. The bar indicates 200 nm.
gradual decrease of fluorescence for 24 h, but actin and soluble cytokeratins showed no significant change for 24 h (Fig. 1D).

Electron microscopic observation of Gal-7 after 3 h of incubation at pH 2.0 showed short, curled fibrillar structures that were not consistent with the morphology of amyloid fibrils (Fig. 1E).

In Vitro Amyloidogenesis of Synthetic Peptides for Gal-7 and Actin—Gal-7 is characterized by 12 \(\beta\)-strand fragments with 5 paired structures (S1/F1, S2/F2, S3/F3, S4/F4, and S5/F5) (Fig. 2A). Seven species of Gal-7 peptides containing two or three sequential \(\beta\)-strand fragments (Gal S1-F2, Gal S3-S4, Gal S4-S5, Gal S3-S5, Gal S5-S6a, Gal F3-F5, and Gal S2-F1) were synthesized (Fig. 2B) and their ThT fluorescence was determined as a function of their incubation times, pH values and doses (\(M\)). Gal S3-S5 and Gal S2-F1 fragments (500 \(M\) each) showed high ThT fluorescence at pH 2.0 after 3 h and 48 h incubations, respectively. After reaching maximal values, Gal S3-S5 and Gal S2-F1 had a gradual decrease during the 24-h and 7-day incubation periods, respectively. Full-length Gal-7 and other fragments, Gal S1-F2, Gal S5-S6a, Gal S3-S4 (not shown), and Gal S4-S5 (not shown) (500 \(M\) each), maintained low values for 7 days (Fig. 3A). The maximal ThT values of Gal S3-S5 and Gal S2-F1 (500 \(M\) each) were obtained at pH 2.0 after 3- and 48-h incubation times, respectively. Other peptides (Gal S1-F2, Gal S5-S6a, Gal F3-F5, Gal S3-S4, and Gal S4-S5), including the full-length Gal-7 molecule, failed to show significant fluorescence in any pH ranges (Fig. 3B). Gal S3-S4 and Gal S4-S5 failed to form amyloid fibrils at pH 2.0 during the 7-day incubation (not shown), indicating that the three \(\beta\)-strand segments (S3, S4, and S5) are essential to forming amyloid aggregates. The dose-dependence of the ThT fluorescence with various doses (10–500 \(M\)) of Gal S3-S5 and Gal S2-F1 fragments at pH 2.0 was determined. Maximal ThT fluorescence of both fragments were increased and the times to reaching equilibrium were shortened in a dose-dependent manner (Fig. 3C). Incubation of Gal S3-S5 (left panel) and Gal S2-F1 (right panel) fragments at pH 2.0 for 3 and 48 h, respectively, produced uniform non-branching needle-like structures with 15–20 nm width and varied lengths (mostly ~300 nm) that were consistent with amyloid fibrils based on the electron microscopy definitions (37) (Fig. 3D).

Synthetic peptides containing the \(\beta\)-strand domain (Leu8-Pro38, Val35-Lys68, Gly150-Leu178, and Lys238-Ile250) of the actin molecule did not have a significant elevation of ThT fluorescence at any pH for 48 h (not shown).

Aggregation of Various Analogs of Gal S3-S5 (Ser31-Gln67) and Gal S2-F1 (Arg120-Phe136) Segments—We further prepared various analogs of Gal S3-S5 and Gal S2-F1 fragments to know the sequence required for amyloidogenic potential. The peptides that were analogous to Gal S3-S5 (Ser31-Gln67), Phe33-Lys65, Ser32-Lys65, and Arg32-Glu66, showed relatively lower amyloidogenic potentials than those from the Ser31-Gln67 fragment for the initial 18 h maintaining the high ThT values for 7 days (not shown), whereas Phe33-Asn63, Val35-Lys65, and His34-Lys65 exhibited an increased ThT level for 3, 5, and 7 days after a lag time of 1 day. Phe33-Gln67 had the lowest amyloidogenic potential for the 7-day incubation (Fig. 4A, right panel). The fragments with a high ThT fluorescence were found to have varied appearances that depended on the peptide size and amino acid residues at both the N- and C- terminal ends by electron microscopy (Fig. 4B). The Leu121-Phe136 and Leu121-Arg134 fragments showed...
considerable amyloidogenic potentials with lower ThT levels than Arg\textsubscript{120}-Phe\textsubscript{136}, while Arg\textsubscript{120}-Ile\textsubscript{135} and Leu\textsubscript{121}-Ile\textsubscript{135} had the lowest amyloidogenic activity (Fig. 4\textsubscript{C}, right panel). Amyloid fibrils with various morphologies that were formed from Arg\textsubscript{120}-Phe\textsubscript{136}, Leu\textsubscript{121}-Arg\textsubscript{134}, and Leu\textsubscript{121}-Phe\textsubscript{136} were observed on an electron microscope (Fig. 4\textsubscript{D}).

**Extension Reaction of Ser\textsubscript{31}-Gln\textsubscript{67} and Phe\textsubscript{33}-Lys\textsubscript{65} Aggregates—** Kinetic studies of the extension reaction focused on the Ser\textsubscript{31}-Gln\textsubscript{67} and Phe\textsubscript{33}-Lys\textsubscript{65} peptides. When soluble Ser\textsubscript{31}-Gln\textsubscript{67} (10 or 50 μM) and sonicated Ser\textsubscript{31}-Gln\textsubscript{67} aggregates, f(Ser\textsubscript{31}-Gln\textsubscript{67}) (2.5 or 5 nmol/100 μl), were incubated at pH 2.0 for 3, 6, 12, and 18 h, the intensities of ThT fluorescence became higher than those of Ser\textsubscript{31}-Gln\textsubscript{67} alone (see Fig. 3\textsubscript{C}, left panel) and f(Ser\textsubscript{31}-Gln\textsubscript{67}) alone (Fig. 5\textsubscript{A}, left panel). No significant increase of ThT fluorescence was observed at pH 4.0, 6.0, 8.0, and 10.0 (not shown). When the Gal-7 molecule (10 or 50 μM) was incubated with sonicated f(Ser\textsubscript{31}-Gln\textsubscript{67}) (5 nmol/100 μl) at pH 2.0 for 3, 18, 30, and 48 h, no increase in fluorescence was observed (Fig. 5\textsubscript{A}, middle panel). The combination of soluble Phe\textsubscript{33}-Lys\textsubscript{65} (10 or 50 μM) and f(Phe\textsubscript{33}-Lys\textsubscript{65}) (2.5 or 5 nmol/100 μl) at pH 2.0 also gave higher ThT values than the individual soluble or fibril forms (Fig. 5\textsubscript{A}, right panel). Electron microscopic studies demonstrated...
that the amyloid fibrils that formed after incubation with the soluble form (Ser31-Gln67 or Phe33-Lys65 peptide) at pH 2.0 appeared to be extended (Fig. 5B) compared with the fibrils that were composed of their respective monomers (See Fig. 4A).

Modulation of Amyloidogenic Peptides of Gal-7 (Ser31-Gln67, Phe33-Lys65, Ser31-Lys65, Arg32-Glu66, Phe33-Asn63, Val35-Lys65, His34-Lys65, and Phe33-Gln67) by Gal-7 Peptides, Actin, and Cytokeratin Fragments—Because Gal-7, actin, and cytokeratins have been identified as the major constituents of amyloid deposits of PLCA, we studied the association of amyloidogenic fragments of Gal-7 with these constituents.

Amyloidogenesis of Ser31-Gln67 at an amyloidogenic dose (200 μM) remained unchanged in the presence of Gal-7 fragments (Gal S3-S5, Ser31-Gln67, Phe33-Lys65, Ser31-Lys65, Arg32-Glu66, Phe33-Asn63, Val35-Lys65, His34-Lys65, and Phe33-Gln67) were synthesized (left panel). The peptides (200 μM) were incubated in 50 mM citrate buffer, pH 2.0 containing 100 mM NaCl at 37 °C for 3, 6, and 18 h and 1, 3, 5, and 7 days (right panel). The peptides with rapid increase of ThT at an early time (3–6 h) in the left panel were excluded from the right panel. B, electron micrographs of amyloid fibrils produced by Gal S3-S5 analogs. Bars: 200 nm.

C, peptides analogous to Gal S2-F1 (Arg120-Phe136), Leu121-Arg134, Arg120-Ile135, Leu121-Ile135, and Leu121-Phe136 were synthesized (left panel). The peptides (500 μM) were incubated in 50 mM citrate buffer, pH 2.0 containing 100 mM NaCl at 37 °C for 0.5, 1, 2, 3, 5, and 7 days (right panel). D, electron micrographs of the amyloid fibrils produced by Gal S2-F1 analogs. Bars indicate 200 nm.

FIGURE 4. Comparison of amyloidogenic potentials of Gal S3-S5 (Ser31-Gln67) and Gal S2-F1 (Arg120-Phe136) analogs. A, peptides analogous to Gal S3-S5 (Ser31-Gln67), Phe33-Lys65, Ser31-Lys65, Arg32-Glu66, Phe33-Asn63, Val35-Lys65, His34-Lys65, and Phe33-Gln67, were synthesized (left panel). The peptides (200 μM) were incubated in 50 mM citrate buffer, pH 2.0 containing 100 mM NaCl at 37 °C for 3, 6, and 18 h and 1, 3, 5, and 7 days (right panel). The peptides with rapid increase of ThT at an early time (3–6 h) in the left panel were excluded from the right panel. B, electron micrographs of amyloid fibrils produced by Gal S3-S5 analogs. Bars: 200 nm.

C, peptides analogous to Gal S2-F1 (Arg120-Phe136), Leu121-Arg134, Arg120-Ile135, Leu121-Ile135, and Leu121-Phe136 were synthesized (left panel). The peptides (500 μM) were incubated in 50 mM citrate buffer, pH 2.0 containing 100 mM NaCl at 37 °C for 0.5, 1, 2, 3, 5, and 7 days (right panel). D, electron micrographs of the amyloid fibrils produced by Gal S2-F1 analogs. Bars indicate 200 nm.
length actin, which was not true for Leu8-Pro32 peptide (Fig. 6A, middle panel). The ThT levels of Lys238-Ile250 and the actin molecule as well as other β-strand peptides of actin (Leu8-Pro32, Val35-Lys68, and Gly156-Leu178) were constantly low for 18 h (not shown). Tryptic digests of insoluble cytokeratins (20–200 μM) inhibited the aggregation of Ser31-Gln67 (200 μM) in a dose-dependent manner (Fig. 6A, right panel). Phe33-Lys65, an analogous peptide of Ser31-Gln67, gave similar results as Ser31-Gln67 (not shown).

We examined the effects of the proteins (Gal-7, actin, and cytokeratins) on the amyloidogenic peptides, Arg120-Phe136, Leu121-Arg134, and Leu121-Phe136, at their non-amyloidogenic dose (100 μM). Amyloidogeneses of Arg120-Phe136 and its analogs Leu121-Arg134 were increased in the presence of the Gal F3-F5 (Gly84, Arg113) fragment, a putative tryptic peptide, but this process was not increased in the presence of other Gal-7 fragments (Gal S1-F2 and Gal S5-S6a) or actin molecule and cytokeratin digests (100 μM each) (Fig. 6B, left and right panels). Amyloidogenesis of Leu121-Phe136 was also increased in the presence of Gly84, Arg113 to the same extent as Arg120-Phe136 and Leu121-Arg134 (not shown). Electron microscopic observation revealed that typical amyloid fibrils with extended filamentous structures, compared with those composed of Arg120-Phe136, Leu121-Arg134, or Leu121-Phe136 alone (see Fig. 4D), were produced by the combinations of Arg120-Phe136/Gal F3-F5 and Leu121-Arg134/Gal F3-F5 (Fig. 6C).

In Vitro Amyloid Fibril Formation of Protease-digested Fragments of Gal-7—Because proteolytic fragments of amyloid precursor proteins such as Aβ and β2-microglobulin have been shown to be more amyloidogenic than intact proteins (39, 40), we re-examined the amyloidogenesis of the degradation products of three candidate proteins (Gal-7, actin, and insoluble cytokeratins) that are produced by the digestion with major apoptosis-related proteases of keratinocytes, including trypsin, cath D, and caspase 3. The degradation patterns of these proteins by trypsin, cath D, and caspase 3 are shown in Fig. 7A. Incubation of the degradation products of Gal-7 at pH 2.0 obtained after 3-h trypsin treatment showed increased ThT fluorescence during the 72-h incubation, but the degraded fragments after 1- and 8-h trypsin treatments did not (Fig. 7B). Incubation of the degradation products of Gal-7 at pH 2.0 obtained after 3-h trypsin treatment showed increased ThT fluorescence during the 72-h incubation, but the degraded fragments after 1- and 8-h trypsin treatments did not (Fig. 7B).
ThT fluorescence (not shown). Electron microscopy of samples taken from 3-h trypsin digests after 72-h incubation at pH 2.0 showed straight non-branching amyloid-like structures (Fig. 7C), which was not the case for other samples from 1- and 8-h trypsin digests (not shown).

**DISCUSSION**

**Amyloidogenesis of Gal-7, Actin, and Cytokeratin Molecules—** Determination of the amyloidogenic potentials of Gal-7, actin, and cytokeratin molecules in vitro revealed that Gal-7 alone showed an increase in fluorescence at an acidic pH (pH 2.0), and the increase was not enough to form typical amyloid fibrils, which is possibly because of the incomplete steric interaction between the β-strand peptides of the Gal-7 molecule (41, 42). Specific short peptides of amyloidogenic proteins, rather than full-length molecules, easily aggregate to form amyloid fibrils. For instance, in Alzheimer disease highly amyloidogenic peptides 1–40 and 1–42 are processed from non-pathological amyloid precursor protein (APP) (43), and, in β2-microglobulin of dialysis amyloidosis, specific peptides (Ser20-Lys41 or Asp59-Thr71) form amyloid fibrils more readily than intact β2-microglobulin molecule (33, 40, 44). This suggests that the fragments of Gal-7, actin, or cytokeratins, even though their full-length molecules have little to no amyloidogenic properties, may exhibit strong amyloidogenesis. To examine this possibility, the amyloidogenic potentials of 1) synthetic peptides with β-strand fragments of Gal-7 and actin, and 2) proteolytic fragments of candidate proteins (Gal-7, actin, and cytokeratins)
produced by apoptosis-related proteases (trypsin, cath D, and caspase-3) were determined.

Amyloidogenesis of Synthetic Peptides—Of the synthetic peptides of Galectin-7 molecule, Gal S3-S5 (Ser31-Gln67) and Gal S2-F1 (Arg120-Phe136) showed strong amyloidogeneses at pH 2.0 with the lag time of 3 h and 48 h, respectively (see Fig. 3C). Kinetic studies on aggregation using various Ser31-Gln67 analogs showed that the Phe33-Lys65 and Ser31-Lys65 variants that lack two amino acid residues at both ends and at the C-terminal end, respectively, were capable of maintaining their amyloidogenic potential, whereas Phe33-Gln67 lacking N-terminal two amino acid residues did not express any amyloidogenesis at least within 7 days (see Fig. 4A). This indicates that 1) Ser31-Gln67 has the highest aggregation activity, 2) Phe33-Lys65 seems to be the minimum sequence required for maintaining a high aggregation potential, because variants of Phe33-Lys65 lacking one or two amino acid residues at N-terminal (His34-Lys65 and Val35-Lys65) or C-terminal end (Phe33-Asn65 and Phe33-Ser64) all show lower aggregation potentials, and 3) the N-terminal two amino acid residues of Ser31-Gln67 (Ser31-Arg32) seem to have a promoting potential whereas C-terminal two residues (Glu66-Gln67) have an inhibiting activity, when variants of Ser31-Gln67 lacking two amino acid residues at N- or C-terminal end (Phe33-Gln67, Ser31-Gln67, and Ser31-Lys65) are compared.

Among the analogs of the Gal S2-F1 fragment (Arg120-Phe136), Leu121-Phe136, and Leu121-Arg134 were amyloidogenic, compared with Arg120-Ile135 and Leu121-Ile135, for the 7-day incubation, indicating that the C-terminal amino acid residue of Arg120-Phe136 is important for amyloidogenesis. It is of particular interest that amyloidogenic peptides, Phe33-Lys65, Leu121-Phe136, and Leu121-Arg134, are all putative tryptic peptides (See Fig. 2B); therefore, several amyloidogenic fragments may be released after the digestion of Galectin-7 with trypsin-like enzymes, although the tryptic cleavage sites Arg54 and Arg134 are located in the Phe33-Lys65 and Leu121-Phe136 peptides, respectively, and their susceptibilities to the trypsin-like enzymes of keratinocytes are unknown. These in vitro studies using synthetic peptides suggest that tryptic degradation of Gal-7 will play a major role in amyloidogenesis of PLCA.

Amyloidogenesis of Proteolytic Fragments—We next examined whether the mixture of degraded peptides of Gal-7 obtained after trypsin digestion (1–8 h) might be amyloidogenic. Galectin-7 fragments produced by trypsin under the restricted condition of 3 h digestion were amyloidogenic. The digestion time-dependent amyloidogenesis suggests that the susceptibilities of the cleavage sites (a total of 16 sites) of Gal-7 to trypsin are not the same. The K and R residues (Lys7, Arg21, Arg23, Arg54, Lys65, Arg75, Lys99, Arg111, and Arg134)
located inside the β-sheet domains will be relatively resistant to trypsin because the tertiary structure of β-sheet may inhibit the access of the enzyme compared with Arg^{83}, Arg^{113}, Arg^{118}, and Arg^{120}, which are located outside the β-sheet domains (See Fig. 2B). Except Arg^{15} and Arg^{72}, which are difficult to cleave because their C-terminal amino acid residues are acidic (P and E, respectively) (45). During the short incubation time (3 h), the peptides, Gly^{84}–Arg^{113} and Leu^{121}–Phe^{136}, of which tryp tic cleavage sites are outside the β-sheet domains will be released; the latter peptide is shown to be amyloidogenic and the former peptide, although not amyloidogenic by itself, potentially stimulates Leu^{121}–Phe^{136} to form amyloid fibrils (see Fig. 6B). As digestion progresses (8 h), the tryp tic sites inside the β-sheet domains begin to be cleaved, resulting in the degradation of the Gly^{84}–Arg^{113} peptide by the tryp tic sites (Lys^{99} and Arg^{111}) located inside the peptide and the production of Leu^{121}–Arg^{134} peptide with a relatively low amyloidogenic potential (see Fig. 4C). These hypotheses seem to explain well why limited trypsin treatment for 3 h produces amyloid fibrils. Keratinocytes do not express pancreatic type trypsin (trypsinogens 1 and 2), but they do produce trypsinogens 4 and 5 and KLKs. Their functions and substrate specificities have not been fully elucidated, but among them, KLK 4, 5, 6, and 8 are known to exhibit trypsin-like specificity (17, 18). Although the identification of the tryp tic enzymes involved in Gal-7 degradation in vivo and the possibility of the involvement of other species of caspases and cathepsins still remain to be determined, the present studies suggest that at least limited degradation of Gal-7 by tryp tic enzymes is involved in the production of amyloidogenic fragments in PLCA.

Amyloid Fibril Extension—We used Ser^{31}–Gln^{67} and Phe^{33}–Lys^{65} fragments in the extension studies. Amyloidogenic peptides, Ser^{31}–Gln^{67} and Phe^{33}–Lys^{65} monomers were both capable of depositing on their respective preformed aggregates and of elongating the amyloid fibrils, suggesting that amyloid fibrils consisting of Ser^{31}–Gln^{67} and Phe^{33}–Lys^{65} peptides are formed via two steps, seed formation (the assembly of monomeric amyloid protein into a template) and extension (the growth of the template by depositions of additional monomeric amyloid protein) (20). However, incubation of the intact Gal-7 molecule in the presence of f(Ser^{31}–Gln^{67}) or f(Phe^{33}–Lys^{65}) failed to increase the rate of elevation of ThT fluorescence. This is in contrast with dialysis amyloidosis in which the combination of the K3 (Ser^{20}–Lys^{41}) peptide seed and intact βM monomer resulted in the extension of amyloid fibrils (33, 38), suggesting that intact Gal-7 cannot be a promoter for amyloid fibril extension and that degradation of Gal-7 is essential for the extension of amyloid fibrils.

Acidified Condition of Apoptotic Cell—The optimal pH for amyloid fibril formation by galectin-7 segments (Ser^{31}–Gln^{67}, Phe^{33}–Lys^{65}, Arg^{120}–Phe^{136}, Leu^{121}–Arg^{134}, etc.) was acidic (pH 2.0), which is not physiological. However, cytoplasmic acidification is now recognized as a general feature of apoptosis in a variety of cell lines, including neutrophils, T-lymphocytes and leukemia cells. Because intracellular acidification, resulting from pH dysregulation, which is in turn due to dephosphorylation of protein exchangers, occurs at early stages of apoptosis (46–48), amyloidogenic galectin-7 segments should be produced by tryp tic enzymes at neutral pH prior to amyloid fibril formation in the intracellular acidified conditions. Previous report that intracellular acidification of apoptosis is preceded by protease (including caspases) activation and that inhibition of the protease activity prevents cytoplasmic acidification in Jurkat T-lymphoblasts (49), strongly suggests a close relationship between apoptosis-induced proteolysis and subsequent cytoplasmic acidification. This supports our hypothesis on the mechanism of amyloid fibril formation in PLCA, which will be discussed for Fig. 8.

Regulation of Amyloid Fibril Deposit—Apoptotic cells will be replaced by amyloid fibrils via complex and multi-step processes. Apoptotic cell death in the epidermis is frequently detected in skin disorders that are unrelated to PLCA, including lichen planus, lupus erythematoses, drug reaction, graft versus host disease (GVHD), and sun-burn (50). Ovoid, PAS-positive eosinophilic apoptotic bodies of various shapes and sizes that have generally been referred to cytoid bodies or Civatte bodies are often observed close to the epidermis or sometimes in the epidermis of these disorders (51), suggesting that apoptotic changes of the epidermis do not always lead to amyloid fibril deposition, but the conversion from apoptotic keratinocytes to amyloid deposition occurs only in the restricted condition. The rate of amyloidogenesis of Gal-7 fragments (Ser^{31}–Gln^{67}, Arg^{120}–Phe^{136}, etc.) was dependent on the content of each Gal-7 fragment (see Fig. 3C), which is controlled by the degree of Gal-7 induction during epidermal apoptosis. We found that limited trypsin treatment (3 h) of Gal-7 was the best condition for the production of amyloidogenic peptides (See Fig. 7B), indicating that appropriate tryp tic enzyme activity is crucial for amyloid fibril formation. Additionally, the amyloidogenesises of Ser^{31}–Gln^{67} and Phe^{33}–Lys^{65} fragments were inhibited by other amyloid constituents (actin and cytokeratin fragments) of PLCA, whereas the amyloidogenic potentials of Arg^{120}–Phe^{136},
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Leu121-Phe136, and Leu121-Arg134 were stimulated in the presence of the Gal F3-F5 fragment (see Fig. 6). Thus, the aggregation of Gal-7 fragments in PLCA is regulated by many factors, including the apoptosis-induced Gal-7 level, activities of apoptosis-related tryptic enzymes and amyloid constituents (Gal-7, actin, and cytokeratins).

**Familial Primary Localized Cutaneous Amyloidosis**—Most cases of PLCA are the non-familial, acquired type, but rare cases of familial PLCA (FPLCA) with autosomal-dominant inheritance have been reported (52). Recent genetic analysis has revealed missense mutations in the oncostatin M receptor-β (OSMR) or interleukin-31 receptor A (IL31RA) gene, which can form a heterodimeric receptor together through IL31 signaling. OSMRβ or IL31RA signaling is related to the reduction of signal for keratinocyte apoptosis (53); therefore, genetic defects in the signaling will induce keratinocyte apoptosis. The mechanism by which these mutations are related to Gal-7 is unknown. The subcellular localization of Gal-7 is variable; it can be located in the intracellular or extracellular space and sometimes on the plasma membrane (22), depending on the cellular conditions. Gal-7 may be directly or indirectly involved in the OSMRβ/IL31RA signaling pathway during keratinocyte apoptosis (28), resulting in the abnormal metabolism of Gal-7 in FPLCA keratinocytes.

Finally, the basic mechanism for promoting amyloid fibril formation in PLCA may be summarized as follows (Fig. 8): 1) induction of keratinocyte apoptosis by UV irradiation and more, 2) induction of Gal-7 expression and activation of apoptosis-related proteases, 3) production of amyloidogenic peptides of Gal-7 by apoptosis-related proteases, including trypsin-like enzymes at neutral pH, 4) aggregation of amyloidogenic peptides in the acidic condition, 5) the aggregation is modulated by apoptosis-related proteases, including trypsin-like enzymes at neutral pH, and 6) growth of amyloid fibrils by deposition of additional Gal-7 fragments.

This study is the first step for understanding the pathogenesis of PLCA. To confirm the amyloidogenic mechanism of Gal-7, further studies will be necessary to identify the species of Gal-7 peptides that accumulate in the lesional skin of PLCA. The roles of actin and cytokeratins in amyloid fibril formation of PLCA also should be clarified.

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