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Identification of an amyloidogenic region on keratoepithelin via synthetic peptides

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Abstract **Mutations of keratoepithelin (KE) gene in human chromosome 5q31 have been linked with corneal epithelial or stromal dystrophies characterized by the abnormal deposits of amyloid fibrils and/or non-amyloid aggregations in corneal tissue. We report herein that synthetic peptide containing amino acid (a.a.) residues of 515–532 of native KE protein can readily form β-sheet-containing amyloid fibrils *in vitro*. Amyloid fibrils formed in various conditions from short synthetic peptides (containing a.a. 515–532 and 515–525, respectively) were characterized by thioflavin T (ThT) fluorescence assay, Congo red staining, electron microscopy (EM) and circular dichroism (CD). Triple-N-methylation of the synthetic peptides prevented the β-sheet polymerization and related amyloid fibril formation. Comparison study with ThT fluorescence further demonstrated that synthetic peptides containing corneal dystrophy-related mutations within this region formed amyloid fibrils to various extents. Our results suggest that each individual dystrophy-related mutation by itself does not necessarily potentiate amyloid fibril formation of KE. Roles of these intrinsically amyloidogenic foci in abnormal KE aggregations and amyloid deposits of stromal corneal dystrophies await further investigation.**

Keywords: Keratoepithelin; Amyloid fibrils; Amyloidogenesis; Corneal dystrophies; Synthetic peptide; Transforming growth factor beta 1

1. Introduction

Corneal stromal dystrophies are a diverse group of hereditary disorders associated with abnormal protein deposits in corneal tissue. Mutations of keratoepithelin (KE), an extracellular matrix protein that was first identified as a transforming growth factor beta 1 (TGFβ-1)-induced protein [1,2], have been linked to at least 13 clinically and histopathologically distinct autosomal dominant corneal dystrophies [3–5]. KE consists of 683 amino acids, containing an amino-termial secretory signal peptide sequence, four ''Fas-like'' domains (~140 amino acids for each domain) homologous to Drosophilia fasciclin-1 (Fas-1), and an arginine–glycine–aspartate (RGD) motif in the C-terminus [1]. KE has been shown to interact with various extracellular matrix proteins [6–9] and promote cell attachment, migration, proliferation and differentiation, likely via the interaction with integrins [8,9]. Abnormal protein deposits in the forms of amyloid fibrils and/or non-amyloid amorphous aggregations have been found in these KE-related corneal dystrophies. Although molecular genetics have unequivocally established the role of KE gene mutations in human chromosome 5q31 for these corneal dystrophies, the pathogenic mechanisms responsible for abnormal protein aggregations remains inconclusive.

Preliminary proteomic studies have suggested that the abnormal aggregations phenotypic for corneal dystrophies may be caused by the aberrant turnover and degradation of mutant KE proteins. When compared with normal corneas, KE proteins extracted from dystrophic corneas with various mutations (R124C, R124H and R124L) were noted for the distinct degradation patterns [10]. In addition, accumulation of a C-terminal KE fragment was found in the dystrophic corneas with a F540S mutation [11]. Since cornea remains the only tissue affected by KE mutations [12], other innate characteristics besides the protein catabolism/degradation are also likely to contribute to the cornea-specific abnormal aggregation of mutant KE proteins, as suggested by other authors previously [13–15].

Amyloidogenesis could also be instigated by intrinsic domains that are either highly amyloidogenic by nature or prone to interconvert toward β-sheet polymerization upon mutations, protein unfolding, proteolysis or under other conducive conditions. Using a synthetic peptide and *in vitro* dialysis approach, it was shown that peptides with mutations of the R124 (R124C, R124H, R124S and R124H) displayed enhanced amyloid fibril formation compared to WT peptide which correlated well with the clinical findings in corneal dystrophy patients [16]. However, discrepancy does exist between the clinical observations and experimental results. When expressed in transfected corneal epithelial cells and CHO cells, WT and mutant KE proteins all displayed similar degradation patterns by immunoprecipitation and Western blots [17,18]. While KE mutations are confirmed in most (but not all) of the dystrophic corneas, both wild-type (WT) and mutant recombinant proteins can form the phenotypic amyloid fibrils *in vitro* [17], indicating that KE proteins are prone to aggregate. This notion is further supported by the recent report of WT KE aggregations in non-dystrophic corneas with secondary amyloidosis [15]. It was also later determined that a shorter peptide with the native sequence missing R124 residue actually had a much higher capability to form amyloid fibrils *in vitro* [13].

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Abbreviations: KE, keratoepithelin; CD, circular dichroism; TGFβ-1, transforming growth factor beta 1; ThT, thioflavin T

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Although the 4th Fas-1 domain near the C-terminus contains a majority of keratoepithelin mutations (such as mutations of L518, L527 and R555) linked with various corneal dystrophies [4], the amyloidogenicity of this domain has not yet been reported. In this study, we investigated amyloid fibril formation of synthetic peptides containing mutation loci of KE-related corneal dystrophies located in the 4th Fas-1 domain and have found a previously unreported amyloidogenic region that can potentially contribute to the abnormal aggregations of KE. We further demonstrated that the amyloid fibril formation of this region could be prevented by N-methylation of the synthetic peptides. The implications of our findings in KE-related corneal dystrophies were also discussed.

2. Materials and methods

2.1. Peptide synthesis

All peptides used in this study were custom-synthesized by the Microchemical facilities at the University of Minnesota using standard solid phase Fmoc amino acid chemistry and Fmoc-Met Wang resin. The crude products were further purified by reverse phase high pressure liquid chromatography with final purity greater than 95% and the sequences were confirmed by fast atom bombardment mass spectrometry (FAB-MS) and/or matrix-assisted laser desorption mass spectrometry (MALDI-MS).

2.2. Peptide solutions for amyloid fibril formation

Stock solutions of synthetic peptides were prepared by dissolving each peptide in DMSO to a concentration of 5 mM. Unless mentioned otherwise, the stock solutions were further diluted into aqueous solutions and incubated at 37 °C for 24 h for amyloid fibril formation experiments. For the pH-dependent amyloid fibril formation, KE18-WT (Fig. 1a) was diluted with solutions ranging from pH 4 to 11, containing 150 mM NaCl and 50 mM of sodium citrate, MES, MOPS, Tris, CHES and CAPS. For the ionic strength-dependent amyloid fibril formation, KE18-WT was diluted with 10 mM Tris, pH 7.0, buffers with various concentrations of NaCl (0, 50, 150, 250, 500, 1000, 2000 and 4000 mM). Due to the difference in critical concentrations for each peptide to form amyloid fibrils *in vitro*, a higher concentration of 0.4 mM peptide was chosen based on a previous report [13] to compare WT with mutant peptides.

2.3. Thioflavin T (ThT) fluorescence

ThT fluorescence assays were conducted according to previous report [13,16,19]. 50 μL samples from *in vitro* fibril formation experiments was mixed with 450 μL of 100 μM ThT in 50 mM glycine–NaOH, pH 8.5, and measured immediately. Scanned fluorescence spectra and changes in ThT emission were obtained either with a FluoroMax-II spectrofluorometer (Jobin Yvon-SPEX, Edison, NJ, USA) using an excitation wavelength at 450 nm scanning from 460 to 600 nm in a 0.3 cm cuvette at 25 °C, or with a fmax-II fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA) using a 444 nm/485 nm filter set ($\varepsilon_x/\varepsilon_m$) in 96-well plates. For ionic strength- and pH-dependent amyloid fibril formation, the formed amyloid fibrils were first pelleted by a TL-100 ultracentrifuge (Beckmen Coulter, Fullerton, CA, USA) at 25000 rpm (38500 × *g*) and then re-suspended in 500 μL ThT solution, as the fluorescence emission of ThT can be affected by experimental conditions related to pH or ionic strength. The formed fibrils from KE18-WT and mutant peptides in the comparison study were also centrifuged and then resuspended in ThT solution as described above.

2.4. TEM and Congo red staining

Negative-stain electron microscopy was used to study the density and morphology of the fibrils formed under various conditions. Peptide samples (~5 μl) were applied onto carbon-coated copper grids and stained with 2% phosphotungstic acid at room temperature for two minutes. Excess solution was blotted away with filter paper and air-dried. The peptide aggregates were examined with a JEOL 1200 transmission electron microscope (TEM) at the Characterization Facility at the University of Minnesota with an accelerating voltage of 80 keV to avoid sample damage caused by high-energy electron beam.

A 10 μl aliquot of samples containing formed fibrils was applied to gelatin-coated glass slides and allowed to air-dry. The slides were then stained with Congo red (1 mM) in 1× TBS, rinsed once in water to remove excess stain and observed with a Zeiss Axiovert 200 light microscope for birefringence under polarized light.

2.5. Circular dichroism (CD)

The far-UV CD spectra were measured with a Jasco J-710 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) to investigate the interconversion of the secondary structures of synthetic peptides. To avoid the interference from DMSO, peptide stocks were made in 150 mM NaCl instead and then adjusted to the appropriate pH. Each peptide spectrum was measured using the average of 10 scans with blank-subtraction and plotting by Kaleidagraph software (Synergy Software, Reading, PA, USA).

3. Results

3.1. The amyloid fibril formation of KE18-WT peptide

In our *in vitro* fibril formation experiments, synthetic peptide KE18-WT (Fig. 1a) readily formed amyloid fibrils when incubated at 37 °C in 1× TBS (150 mM NaCl, 10 mM Tris–HCl, pH 7.0). When compared with ThT only or the control peptide with a scrambled sequence (“KE18-Scrambled”), KE18-WT displayed a significantly higher ThT intensity (Fig. 1b). As measured by the ThT fluorescence intensity, fibril formation increased from 125 μM of KE18-WT to 0.4 mM in a concentration-dependent manner. No fibrils were found at concentrations below 50 μM (data not shown). The aggregations of KE18-WT peptide were shown to be of amyloidogenic nature as demonstrated by the Congo red staining with apple-greenish birefringence when observed under polarized light microscope (Fig. 1c). By electron microscopy, KE18-WT peptides formed extensive fibrils of several hundreds nm in length with diameters ranging from 5 to 10 nm (Fig. 1d). Spheres (non-fibril aggregates) of various sizes (15–45 nm in diameter) were also observed along with those fibrils. When KE18-WT peptides (125 μM) were incubated for 24 h in conditions favorable for fibrils formation (such as pH 7.0) or conditions unfavorable for fibril formation (pH 9.0, Fig. 2b), two findings were noted by the far-UV CD spectroscopy (Fig. 1e): (1) a large negative peak around 200 nm in pH 9.0 shifted toward the longer wavelength and decreased in amplitude in pH 7.0; (2) an increase of the negative ellipticity around 220 nm was also noted from pH 9.0 to pH 7.0. The CD spectrum of KE18-WT in pH 7.0 before incubation (0 h) is similar to that of pH 9.0 after 24 h incubation. These findings indicated clear transition from random coils in pH 9.0 to orderly secondary structures such as β-sheets in pH 7.0.

3.2. Characterization of the amyloidogenesis of KE18-WT

The kinetics of amyloid fibril formation and the effects of ionic strength and pH on the fibril formation of KE18-WT were further characterized *in vitro*. Fluorescence intensities of ThT at 485 nm were measured for amyloid fibrils formed by KE18-WT (125 μM) under various conditions. The amyloid fibril formation progressed relatively fast initially and reached plateau at 24 h under our testing condition (Fig. 2a). No fur-