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Human alpha A-crystallin missing N-terminal domain poorly complexes with filensin and phakinin

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ABSTRACT

The aim of this study was to determine relative importance of N-terminal domain and C-terminal extension of αA-crystallin during their *in vitro* complex formation with phakinin and filensin (the two lens-specific intermediate filament [IF] proteins). Cloned phakinin, filensin and vimentin were purified under a denaturing conditions by consecutive DEAE-cellulose-, hydroxyapatite- and Sephadex G-75-column chromatographic methods. WTαA-crystallin, αA-NT (N-terminal domain [residue number 1−63])-deleted and αA-CT (C-terminal terminal extension [residue number 140−173]-deleted), were cloned in pET 100 TOPO vector, expressed in BL-21 (DE3) cells using 1% IPTG, and purified using a Ni^{2+}-affinity column. The following two *in vitro* methods were used to determine complex formation of WT-αA, αA-NT, or αA-CT with phakinin, filensin or both phakinin plus filensin together: an ultracentrifugation sedimentation (centrifugation at 80,000 × g for 30 min at 20 °C) followed by SDS-PAGE analysis, and an electron microscopic analysis. In the first method, the individual control proteins (WT-αA, αA-NT and αA-CT crystallin species) remained in the supernatant fractions whereas phakinin, filensin, and vimentin were recovered in the pellet fractions. On complex formation by individual WT-αA-, αA-NT or αA-CT-species with filensin, phakinin or both phakinin and filensin, WT-αA and αA-CT were recovered in the pellet fraction with phakinin, filensin or both filensin and phakinin, whereas αA-NT remained mostly in the supernatant, suggesting its poor complex formation property. EM-studies showed filamentous structure formation between WT-αA and αA-CT with phakinin or filensin, or with both filensin and phakinin together but relatively poor filamentous structures with αA-NT. Together, the results suggest that the N-terminal domain of αA-crystallin is required during in *vitro* complex formation with filensin and phakinin.

1. Introduction

The cytoskeletal proteins of the vertebrate lens include actin-containing microfilaments, microtubules, and two different intermediate filament (IF) networks, one based on vimentin and the other based on copolymers of lens-specific filensin and phakinin. The intermediate filaments name is given because of their diameter of about 10 nm, which is intermediate between thin 7 nm actin filament of and thick 25 nm microtubules. The lens-exclusive IF are known as beaded filaments, and are composed of only two proteins, phakinin (also known as CP49 or CP45) and filensin (also called CP94 or CP115) [1–4]. The ubiquitous IFs have three common structural domains: head, rod, and tail [1–4]. Filensin has a shortened central rod domain, and therefore, is different from the typical structure of proteins in this family. Phakinin lacks the C-terminal tail domain [4] and shows divergence in the highly conserved motif that borders the central helical domain of all the intermediate filaments. The amino acid sequence analysis showed that in filensin, the conserved TYRKLLGEE motif at the end of the rod domain was modified to RYHRIIE (I/N) EG, and the conserved LNDR motif at the beginning of the rod domain was altered to LGER [5]. Filensin and phakinin readily polymerize *in vitro* and form 10 nm heteropolymeric filaments [4,5] at a ratio of 2:1 or 3:1 [5].

Alpha-crystallin has been shown to be a major component of the beaded filaments [8], and is also associated with intermediate cytoskeleton [5–9]. The interaction between αB-crystallin and IF proteins has been studied in details [8,9]. During thermal stress, αB-crystallin selectively targets intermediate filament proteins [9].

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However, presently such interactions between αA-crystallin and filensin and phakinin are not well characterized.

Among lens crystallins (α-, β-, and γ-crystallins), only the α-crystallin belongs to a heat-shock protein family (HSPs), and has chaperone activity [10]. Like other HSPs, α-crystallin also contains a highly conserved sequence of 80–100 residues called the α-crystallin core domain (residue no. 64–139 in αA-crystallin and residue no. 66–144 in αB-crystallin) [11]. Based on similarities with the structures of other HSPs, it is believed that the N-terminal region of both αA- (residue no. 1–63) and αB- (residue no. 1–66) crystallins forms independently folded domains, whereas the C-terminal region (residue no. 143–173 in αA- and no. 147–175 in αB-crystallin) is flexible and unstructured [12]. On the removal of N-terminal residues (partial or 1–56 residues of N-terminus) or the C-terminal extension (partial or 32–34 residues of C-terminus) of αA- and αB-crystallins, the proteins showed improper folding, reduced chaperone activity, and formation of trimers or tetramers [12–15]. The residue no. 42–57 and residue no. 60–71 of αB-crystallin interact with αA-crystallin [16,17], and the pin-array analysis has further shown that five peptide sequences of αB-crystallin (i.e., residue no. 37–54 [in the N-terminal region], residue no. 75–82, 131–138, 141–148 [form β-strand in the conserved α-crystallin domain], and residue no. 155–166 [in the C-terminal extension]) interact with αA-crystallin [18]. In spite of these studies, the regions and individual amino acids in the αA- and αB-crystallins that interact with filensin and phakinin in IF have not been identified.

Our report has shown that the individual N-terminal domain, C-terminal extension or core domain of both αA- and αB-crystallins exhibit varied biophysical properties and chaperone activity [13,14]. Each region was found to retain some level of chaperone function, and most notably, the N-terminal domain of both crystallins showed maximum chaperone activity [13,14].

Our recent results also show that in the lens-specific conditional βA3-knockout model, calpain activation leads to cleavages of β-tubulin, αA-crystallin and phakinin, and their fragments became water insoluble (Unpublished results). Our results further showed that the cleavage of intermediated filament proteins (phakinin and filensin) could occur *in vivo* [19], and might alter lens cytoskeletal structure, and also interactions of αA-crystallin with phakinin and filensin. Recently it was shown that in the lenses from 2-day old αA-R49C and αB-R120G aB-R120G knock-in mutant mice, an increased association between αA-crystallin and filensin relative to age-matched wild type lenses occurs [20]. Additionally, we have also identified complexes of crystallin fragments with fragments of filensin and phakinin in water soluble high molecular weight (WS-HMW)-proteins and water-insoluble proteins of aging and cataractous human lenses [21–23]. The water soluble covalent multimers with $M_r > 90$ kDa, isolated from normal human lenses of 25, 41, 52, and 72-year-old human donors, showed two types of covalent multimers [21]. The first type complex was composed of fragments of 8 different crystallins (i.e., αA, αB, βA3, βA4, βB1, βB2, γS, and γD), and the second type contained α-, β-, and γ-crystallins (possibly fragments) and the two beaded filament proteins (phakinin and filensin). Together, the results suggested that that during normal aging, the crystallin fragments form aggregates among themselves, and also with filensin and phakinin, or with another IF protein (vimentin). The mechanism of such complex formation in context of cataract development tis presently unclear.

To extend our above findings, in this study we have examined *in vitro* interactions of wild-type (WT)-αA, and its two deletion mutants, αA-NT (missing the N-terminal domain, residue no. 1–63) and αA-CT (missing C-terminal extension, residue no. 140–173) with phakinin and filensin. The rationale was that the information would elucidate which region of αA-crystallin is important in interactions with filensin and phakinin. The study showed that the N-terminally truncated αA-crystallin exhibited poor complex formation property with phakinin and filensin, and therefore, this region is required to interact with phakinin and filensin.

2. Materials and methods

2.1. Materials

The molecular weight protein markers were from either GE Biosciences (Piscataway, NJ) or Promega (Madison, WI). Unless indicated otherwise, all other molecular biology grade chemicals used in this study were purchased from Fisher (Atlanta, GA) or Sigma (St. Louis, MO) companies. *E. coli* BL21 (DE3) and BL-21 Ai bacterial strains were obtained from Invitrogen (Carlsbad, CA). The clones for WT-αA, and its N-terminal domain deleted- or C-terminal extension-deleted mutant proteins were generated in our laboratory as previously described [13,14]. The clones for phakinin (CP49), filensin (CP115), and vimentin were generous gift from Dr. Paul FitzGerald, University of California-Davis. Cells were propagated in Luria broth, and recombinant bacteria were selected using ampicillin.

2.2. Protein expression, purification, and assembly

E. coli BL21 (DE3) was transformed with WT-αA and its mutant amplicons using a standard *E. coli* transformation procedure as described previously [13,14]. The WT-αA, N-terminal domain-deleted mutant [αA-NT] and C-terminal extension-deleted mutant [αA-CT] were expressed, and purified as previously described by us [13,14]. Similarly, *E. coli* BL21-Ai was transformed with amplicons of phakinin, filensin, and vimentin using a standard *E. coli* transformation procedure. The intermediate filaments were over-expressed by addition of 0.1% arabinose, and the cultures were incubated further at 37 °C for 6 h. The cells were harvested and resuspended in a lysis buffer [25 mM Tris-HCl (pH 8.0) containing lysozyme (10 mg/mL), 50 mM glucose, and 10 mM EDTA] at room temperature for 30 min. Next, the preparation was lysed by sonication at 5 °C, and DNA was degraded using DNase I (10 μg/ml) and incubation on ice for 30 min. The soluble fraction was separated by centrifugation at 8000 × g for 15 min at 5 °C, and the pellet was resuspended in a detergent containing buffer [0.5% NP-40 and 1 mM EDTA]. The detergent-soluble fraction was separated by centrifugation at 8000 × g for 15 min at 5 °C. Next, the pellet was washed with 10 mM Tris buffer (pH 8.0) containing 1.5 M potassium chloride, 5 mM EDTA, and 0.5% NP-40, centrifuged at 8000 × g for 15 min at 5 °C, and the pellet was washed with 10 mM Tris (pH 8.0) buffer containing 0.15 M potassium chloride, 5 mM EDTA, and 0.5% NP-40. The pellet (recovered after centrifugation at 8000 × g) was washed with 1 mM EDTA and 0.5% NP-40. After a final centrifugation at 8000 × g for 15 min at 5 °C, the pellet was resuspended in an equilibration buffer (8 M urea containing 10 mM Tris (pH 7.5) and 1 mM EDTA [EB Buffer]).

The IF proteins (i.e., phakinin, filensin and vimentin) were purified by modified methods as previously described [24,25] using a DEAE-cellulose column followed by a hydroxyapatite column under denaturing conditions. If needed, the proteins were further purified using a size-exclusion Sephadex G-75 column under denaturing conditions and examined for their purity by SDS-PAGE analysis (Fig. 1A).

2.3. Assembly of filensin or phakinin alone, or together with WT-αA, αA-NT or αA-CT proteins

The IF (0.2–0.4 mg/ml) were assembled *in vitro* at 2:1 M ratio (phakinin or filensin alone or phakinin plus filensin together) with

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