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Differential Binding of Mutant (R116C) and Wildtype AlphaA Crystallin to Actin

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Abstract

Purpose—Quantitate the interaction of mutant (R116C) and wildtype human alphaA crystallins with actin.

Methods—AlphaA crystallins, expressed in a recombinant system, were purified, followed by passage through an actin affinity column.

Results—Binding of mutant alphaA crystallin was significantly less than binding of wildtype alphaA crystallin.

Conclusions—The R116C mutation of alphaA crystallin found in human cataracts binds less to the cytoskeletal component actin. Since both alphaA crystallin and actin are necessary for proper development of the lens, decreased binding of the mutant protein to actin may perturb normal differentiation processes of lens cells which are necessary for transparency.

Keywords

alphaA crystallin mutation; human cataractogenesis

INTRODUCTION

Mutations in lens crystallins have been associated with development of congenital cataracts in humans, and abnormalities in the sequence of these proteins have been found in all the major classes of crystallins (alpha, beta, and gamma crystallins; for a review, see Ref. 1). Expression of the mutant proteins in a recombinant system have demonstrated that relative to wildtype protein, the mutant proteins many times exhibit abnormalities in physiochemical properties including changes in oligomerization, three-dimensional structure, solubility, and molecular chaperone activity.¹ Based upon these *in vitro* studies, it has been suggested that, *in vivo*, the

mutant crystallins may lead to accelerated protein denaturation resulting in precipitation and/or the formation of large aggregates that can directly scatter incident light.

The alpha crystallins are comprised of two proteins, alphaA crystallin and alphaB crystallin, which exhibit extensive sequence similarity.² One of the most well-characterized cataracts has been the R116C mutation of alphaA crystallin, which has been linked to congenital cataracts in two independent pedigrees.^{3,4} Depending upon the age of the individual, the cataracts involved nuclear opacities, as well as cortical and posterior subcapsular opacities. In addition, the mutations were many times associated with microphthalmia.

Arginine-116 of alphaA crystallin is highly conserved in numerous mammalian species, as well as in chicken and frog.⁵ Consistent with the evolutionary conservation of this residue, recombinant expression of alphaA crystallins lacking the positive charge of the guanidine group of arginine resulted in proteins with decreased chaperone activity and increased aggregation.⁶ The decreased chaperone activity could result in decreased protection against denaturation and aggregation *in vivo*, while increased aggregation could directly result in an increase in light scattering.

In addition to its role as a molecular chaperone, numerous studies have also suggested that alphaA crystallin may be involved in normal lens cell development via interaction with the cytoskeletal component actin. Alpha crystallins co-localize with actin microfilaments in lens cells,⁷ and affinity chromatography has demonstrated direct binding of alpha crystallins with actin in a saturable manner.⁸ Polymerized actin is necessary for proper development of lens cells in culture,⁹ and alpha crystallins have been shown to stabilize actin microfilaments against chemically induced depolymerization.¹⁰ Furthermore, when exogenous alpha crystallins were internalized into lens epithelial cells in culture, the resulting cells exhibited morphological characteristics of differentiating cells.¹¹ This effect was not seen when beta or gamma crystallins were used.¹¹ Consistent with the importance of alpha crystallins, a knockout strain of mice lacking expression of both alphaA and alphaB crystallins showed numerous abnormalities in lens cell shape and cell differentiation.¹²

Taken together, the observations suggest that loss of solubility and/or chaperone activity may not be the only mechanism(s) of cataractogenesis occurring in lenses with the R116C mutation, and that possible alterations in the interaction of mutant alpha crystallins with the actin component of microfilaments may also play an important role in abnormal lens development. The following results show that relative to wildtype alphaA crystallin, the R116C mutant shows decreased binding to actin.

MATERIALS AND METHODS

Human recombinant alphaA was expressed as previously described using the following protocol.¹³ Total RNA was extracted from lenses from eyes of a young human donor less than one year of age obtained from the Lions Eye Bank of Oregon and approved by the Oregon Health and Science University institutional review board. The RNA was transcribed into cDNA and then amplified using gene-specific primers to alphaA crystallin. The forward primer contained the sequence 5'-ATG GAC GTG ACC ATC CAG C-3'. The reverse primer contained the sequence, 5'-GAG CCA GCC GAG GCA ATG-3'. This RT-PCR product was inserted into pCR T7/CT TOPO vector (Invitrogen, Carlsbad, CA) and transformed into the cloning vector cells, Top 10F' (Invitrogen). Clones containing the correct insert were confirmed by sequencing and used below for expression. Using the wildtype vector as the template, an Arg at position 116 was replaced with a Cys residue by site-directed mutagenesis by mutating residue 413 in the gene sequence.

Both wildtype and mutant alpha crystallins were expressed in *E. coli* BL21(DE3) cells following induction with 0.5 mM IPTG (isopropylthiogalactoside). After induction, cells were lysed by sonication, and alphaA crystallin was purified using a gel filtration TSK3000SW column (Tosoh Bioscience, Tokyo, Japan), followed by a Uno Q-1 anion exchange column (Bio-Rad Laboratories, Hercules, CA) using a 0–0.5-M linear gradient of NaCl over a period of 30 min. Protein concentration of the purified protein was determined as previously described,¹⁴ using bovine serum albumin as standard. Mass spectral measurements were made on an electrospray ionization mass spectrometer (ThermoFinnigan, San Jose, CA). Observed average mass for wild-type alphaA was 19910.0 compared to the expected mass of 19909.5. The observed mass for the alpha A R116C was 19856.0 compared to the expected mass of 19856.4.

Preparation of actin affinity gel using porcine heart actin (Sigma Chemical Co., St. Louis, MO) and Sepharose 4B gel (Sigma Chemical Co.) was exactly as done previously.⁸ After covalently coupling the actin to the Sepharose beads, 200 μ l of the gel was loaded into an Econo-Column (Bio-Rad Laboratories), followed by washing with 1.0 ml TDN buffer (0.022 M Tris, 0.1 M NaCl, 4.0 mM EDTA, pH 7.4), then incubation with 400 μ l TDN buffer containing 0.04% (v/v) Tween 20 for 15 min at 37°C. AlphaA crystallin (300 μ g in 0.2 ml of TDN buffer) was incubated with the column at 37°C for 16 hr, followed by washing with 1.0 ml of a solution containing 1 M NaCl in TDN buffer. Freshly prepared 8 M urea in distilled water (0.8 ml) was used to elute alphaA crystallin bound to the column.

Aliquots (0.1 ml) of the eluted material were injected into an analytical C₁₈ reverse phase column (Phenomenex, Torrance, CA), and bound alpha crystallin eluted with a linear gradient of 35%–65% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid, over a period of 30 min.

RESULTS

Figure 1 shows the elution profiles of wildtype and mutant alphaA crystallin bound to the actin affinity column. Both wildtype and mutant alphaA crystallins elute at approximately 18 min, but the magnitude of the mutant alphaA crystallin was much less. Different amounts of purified wildtype and mutant alphaA crystallins were also injected into the column, and the area of the peaks used to plot a standard curve. Each of the peaks shown in Figure 1 was within the linear range of binding (results not shown). The standard curves were then used to compute the amount of alphaA crystallin binding to the actin affinity column.

The averages of triplicate determinations of either wildtype or mutant alphaA crystallin binding are shown in Table 1. The wildtype alphaA crystallin binds 1.98 ± 0.13 μ g, while the mutant binds 0.95 ± 0.05 μ g. Statistical analysis using Student's *t*-test showed that the difference in binding was significant ($p < 0.05$).

DISCUSSION

Previous studies of mutated lens crystallins associated with human congenital cataracts have characterized the physiochemical properties of the expressed proteins.¹ These reports showed that the mutant proteins exhibited abnormalities in aggregation, solubility, phase separation, and molecular chaperone properties.

One of the most studied mutations has been the substitution of half-cysteine for arginine at residue 116 of alphaA crystallin.³ The mutation is associated with development of nuclear, cortical, and subcapsular cataracts in two independent human pedigrees.^{3,4} The presence of microphthalmia in many of the affected patients suggests that simple aggregation/insolubilization of alphaA crystallin in differentiated lens fiber cells may not be the only possible mechanism occurring during cataractogenesis, but rather, abnormalities in lens development may also play a role in the opacification process.

The hypothesis is consistent with the results of this study, which showed decreased actin binding of the R116C mutant of alphaA crystallin relative to wildtype alphaA crystallin. Alpha crystallins have been shown to bind to and stabilize polymerized actin,^{7,10} which is necessary for normal differentiation of lens cells.⁹ Based upon the use of actin affinity chromatography, the nature of this interaction is tight binding that is not dissociated upon washing of the column with dilute buffer. However, it is still possible that additional weak binding of actin and alpha crystallin occurs, which can only be determined under equilibrium conditions. Such binding studies are dependent upon knowledge of the relative amounts of actin and alpha present during different stages of lens cell differentiation.

Previous studies have also shown that internalization of alpha crystallins stimulates differentiation of lens epithelial cells in culture.¹¹ Furthermore, a knockout mouse strain that lacks both alphaA and alphaB crystallins develops cataracts with gross abnormalities in lens cell differentiation and shape.¹² A knockout strain that only deletes the alphaA crystallin gene, but not the alphaB crystallin gene, shows microphthalmia and eventual opacity of the lens.¹⁵ In addition, epithelial cells derived from the alphaA crystallin homozygous mouse show increased cell death during mitosis.¹⁶ Together these studies demonstrate the important role of alphaA crystallin in development and maintenance of lens transparency. The abnormalities in differentiation could be the direct result of changes in the properties of the actin filaments, which have been shown to be important for both lens cell shape and differentiation.⁹ Alpha crystallins have also been shown to stabilize actin filaments,¹⁰ and disorganization of these filaments may directly lead to lens opacification.¹⁷ Together, the results suggest that developmental abnormalities, caused at least in part by abnormal binding of alphaA crystallin to actin, should be considered as a possible mechanism in human cataractogenesis.

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FIGURE 1.

Elution profiles of wildtype and mutant alphaA crystallins bound to actin affinity column. Eluent after washing columns with 8 M urea was injected into a C₁₈ reverse-phase column and resolved using a linear gradient of acetonitrile. Arrows designate the peaks used for quantitation of alphaA crystallin binding. Top, wildtype alphaA crystallin; bottom, R116C mutant of alphaA crystallin.

TABLE 1

Binding of wildtype and mutant alphaA crystallin to actin

Sample	AlphaA crystallin bound (μg)
AlphaA crystallin wildtype	1.98 ± 0.13
AlphaA crystallin R116C mutant	0.95 ± 0.05