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Protein-protein interactions involving congenital cataract T5P γ C-crystallin mutant: A confocal fluorescence microscopy study

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Abstract

The human lens crystallin gene CRYGC T5P is associated with Coppock-like cataract and has a phenotype of a dust-like opacity of the fetal lens nucleus and deep cortical region. Previous in vitro mutation studies indicate that the protein has changed conformation, solubility, and stability, which may make it susceptible to aggregation, as seen in cataractous lens and cell culture expression. To investigate the mechanisms leading to these events, we studied protein-protein interactions using confocal fluorescence resonance energy transfer (FRET) microscopy. The method detects protein-protein interactions in the natural environment of living cells. Crystallin genes (CRYGC T5P, CRYGC, and CRYAA) were fused to either the green fluorescence protein (GFP) or red fluorescence protein (DsRED or RFP) vector. Each of the following GFP-RFP (donor-acceptor) plasmid pairs was cotransfected into HeLa cells: γ C- γ C, γ C- γ CT5P, γ CT5P- γ CT5P, α A- γ C, and α A γ CT5P. After culture, confocal fluorescence cell images were taken. Protein-protein interactions in the form of net FRET were evaluated. The confocal fluorescence images show that cells expressing T5P γ C-crystallin contain many protein aggregates, but cells co-expressing with either γ C- or α A-crystallin reduce the aggregation considerably. FRET determination indicates that γ CT5P- γ CT5P shows less protein-protein interaction than either γ C- γ C or γ C- γ CT5P. Cotransfection with α A-crystallin (α A- γ C or α A-T5P γ C) increases nFRET compared with γ C- γ C or γ C-T5P γ C. Our results demonstrate that T5P γ C-crystallin shows more protein aggregates and less protein-protein interaction than WT γ C-crystallin. Chaperone α A-crystallin can rescue T5P γ C-crystallin from aggregation through increased protein interaction. The formation of congenital cataract may be due to reduced protein-protein interactions and increased aggregation from an insufficient amount of α -crystallin for protection.

Keywords

γ C-crystallin; congenital cataract mutation; protein-protein interaction; confocal fluorescence microscopy; fluorescence resonance energy transfer

1. Introduction

Protein-protein interactions play an important role in cellular and protein function. The majority of lens proteins are soluble crystallins, which have been demonstrated to interact by short-range order in the lens, where protein concentration is very high (Benedek 1971; Delaye

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and Tardieu 1983; Bettelheim 1985). At this high concentration, few suitable physicochemical methods can be used. To study relatively low concentrations in solution, various methods, such as light scattering, FT-IR, fluorescence polarization, NMR, microequilibrium, and fluorescence resonance energy transfer (FRET) have been used (Siezen and Owen 1983; Mach et al. 1990; Liang and Li 1991; Koenig et al. 1992; Tardieu et al. 1992; Cooper et al. 1994; Liang and Chakrabarti 1998; Ponce and Takemoto 2005; Liang and Liu 2006). These methods require extensive purification, and proteins may not be under true physiological conditions. To overcome this drawback, we have extended the interaction study to cultured cells with a two-hybrid system assay and confocal FRET microscopy imaging, which have the advantage of not requiring protein purification. We have reported both protein-protein interactions among various crystallins (Fu and Liang 2002b; Liu and Liang 2008) and changes in such interactions due to mutations in some congenital cataracts (Fu and Liang 2003; Liu and Liang 2005; Liu et al. 2007).

The two-hybrid system has one disadvantage absent from confocal FRET microscopy in that it requires two expressed proteins to relocate to the nucleus for transcriptional activation of the reporter gene; a large or insoluble protein may have difficulty entering the nucleus. Our earlier two-hybrid system study involving the T5P γ C mutant may have given an inconclusive result (Fu and Liang 2003) because of the limited solubility of the T5P mutant (Fu and Liang 2002a; Pigaga and Quinlan 2006). Here we further investigate the interaction properties of T5P γ C-crystallin using confocal FRET microscopy.

Confocal FRET microscopy uses two fused proteins to detect protein-protein interactions; we have used a green fluorescence protein (GFP) as the donor and a GFP variant red fluorescence protein (DsRED or RFP) as the acceptor. FRET occurs when the two target proteins interact. Confocal FRET microscopy provides an additional advantage in that it allows direct assessment of protein-protein interactions in the natural environment of living cells (Xia and Liu 2001; van Rheenen et al. 2004; Wallrabe et al. 2006). Our recent studies involving crystallin and membrane MIP26 demonstrate the technique's versatility (Liu and Liang 2008).

2. Materials and Methods

2.1. Subcloning γ C-crystallin and T5P mutant genes to GFP or RFP vector

The γ C- (WT and T5P) and α A-crystallin genes were subcloned into either the pAcGFP-C1 or the pDsRED monomer-C1 (Clontech, Palo Alto, CA) as described previously (Liu and Liang 2008). PCR was performed with the forward primers containing the *xho*I restriction site and reverse primers containing the *Eco*RI site (Table 1). The primers were custom-synthesized (Invitrogen, Carlsbad, CA). All constructs were verified for correct sequences. The resulting constructs were designated GFP- α A, RFP- α A, GFP- γ C, RFP- γ C, GFP-T5P γ C, and RFP-T5P γ C.

2.2. Mammalian cell transfection

Lipofectamine 2000 reagent (Life Technologies, Rockville, MD) was used for transfection into human HeLa cells. In brief, cells were cultured in an MEM medium supplemented with 10% fetal calf serum, 2mM glutamine, and 1% penicillin/streptomycin antibiotics (GIBCO BRL, Gaithersburg, MD) in a 5% humidified CO₂ incubator at 37°C. One day before transfection, HeLa cells (1×10^5 /ml) were seeded into a 35-mm culture dish. After culturing for 24 h to obtain at least 85% confluence, they were cotransfected with appropriate plasmids using lipofectamine 2000 reagent with 5 μ g of each cDNA and 10 μ l of lipofectamine 2000. After incubation for 6 h, the medium was changed and incubation continued up to 48 h for imaging analysis.

2.3. Imaging with confocal FRET microscopy

Confocal images were acquired with a Zeiss Laser Scanning Microscope (LSM) 510 META Axioplan 2 confocal microscope (Carl Zeiss, Inc., Thornwood, NY) at the Optical Imaging Facility of the Harvard NeuroDiscovery Center. Images (12-bit) of multitrack channels with the following wavelengths were recorded: the green channel (donor excitation/donor emission = 475nm/505nm: GFP_{ex/em}), the red channel (acceptor excitation/acceptor emission = 557nm/585nm: RFP_{ex/em}), and the FRET channel (donor excitation/acceptor emission = 475nm/585 nm: FRET_{ex/em}).

2.4. FRET image data analysis

The LSM images were analyzed as described (Liu et al. 2007). Briefly, the intensities of total gray values in the three channels, I_{GFP} (λ_{ex}/λ_{em} = 475/505 nm), I_{RFP} (557/585 nm), and I_{FRET} (475/585 nm), were measured in the same regions of interest (ROI). After adjustment of threshold, the net FRET (I_{nFRET}) values were obtained by correcting contributions from spectral bleed-through (SBT) of the donor and acceptor in the FRET channel with the following equation as described in (Xia and Liu 2001):

$$I_{nFRET} = I_{FRET} - I_{RED} \times a - I_{GFP} \times b \quad (1)$$

where a is the coefficient due to SBT of acceptor signal to the FRET channel and b is the coefficient due to SBT of donor channel to the FRET channel. SBT arises from overlapping of spectra: donor SBT from overlapping of part of the emission spectrum of the donor with emission spectrum of the acceptor, and acceptor SBT from overlapping of part of the excitation spectrum of the acceptor with excitation spectrum of the donor. SBT was determined with cells expressing DsRED construct only (with RED signal but no GFP signal) or AcGFP construct only (with GFP signal but no RED signal) and is defined by the following equations:

$$a = \text{FRET}_{ex/em} / \text{RED}_{ex/em} \quad (2)$$

$$b = \text{FRET}_{ex/em} / \text{GFP}_{ex/em} \quad (3)$$

The contributions from SBT were removed by the computer program. In order to make the values of I_{nFRET} comparable among different ROIs and cells, I_{nFRET} were normalized with I_{GFP} intensity.

2.5. Statistical analysis

Data are expressed as the mean ± SE from a minimum of three independent experiments. Statistical analysis was performed with a Student's t-test, with $p < 0.05$ as the criterion of significance.

3. RESULTS

3.1. Confocal fluorescence images

There were more protein aggregates in the cells transfected with T5P γC-crystallin than with WT γC-crystallin. Typical LSM images are shown in Fig. 1; cells expressing T5P γC-crystallin contain more and larger protein aggregates than cells of expressing WT γC-crystallin. Protein aggregates were seen as bright, dense spots; their fluorescence intensities are saturated in LSM images. The percent of cells with protein aggregates is almost four-fold greater in cells

transfected with T5P γ C-crystallin than in cells transfected with WT γ C-crystallin (Fig. 2) ($p < 0.05$). Cotransfection with α A-crystallin gene reduced the aggregation by almost six-fold ($p < 0.001$). Cells with protein aggregates were counted regardless of the size or number of protein aggregates in all LSM images.

3.2. FRET analysis

We used the following GFP-RFP donor-acceptor pairs for studying protein-protein interaction: γ C- γ C, γ C-T5P γ C, T5P γ C-T5P γ C, α A- γ C, and α A-T5P γ C. The typical LSM images are shown in Fig. 3, revealing that all paired crystallins interact with one another, as shown in the nFRET images in pseudocolor. The first three columns are images of green, red, and FRET channel, respectively. Green images are much brighter than red images; this may be because of the lower brightness of red fluorescence protein rather than because of lower expression levels. The FRET images also showed contribution from SBT, which were removed according to equation 1. The pseudocolor images show the variations in gray levels (pixel densities) in color for clearer indication of the extent of energy transfer and thus protein-protein interaction.

The nFRET values were normalized with green fluorescence (Fig. 4). Fifty to eighty ROIs were selected for each pair of fusion proteins. ROIs were chosen in the areas that contain no protein aggregates. The T5P γ C-T5P γ C pair shows a significantly less normalized nFRET value than either γ C- γ C or γ C-T5P γ C pair ($p < 0.001$). When replacing γ C-crystallin or T5P γ C-crystallin with α A-crystallin in the pairs of γ C- γ C or γ C-T5P γ C, normalized nFRET values increase significantly ($p < 0.001$), and the difference between the pairs α A- γ C and α A-T5P γ C is also significant ($p < 0.001$).

4. DISCUSSION

The T5P γ C-crystallin mutant is one of the cataract crystallin genes that reflects a clear relationship between the cataract genotype and the corresponding phenotype (Heon et al. 1999). Its reported low solubility, loss of stability to heat, and decreased protein-protein interaction with other crystallins may underlie the formation of cataract (Fu and Liang 2002a; 2003; Liang 2004; Pigaga and Quinlan 2006). The observation that the mutation causes the loss of protein-protein interaction is particularly interesting and important. Protein complex formation is the key to protein/cell function. In the lens, protein interactions among various crystallins enhance protein stability. The most prominent example is the α A- and α B-crystallin subunit interaction that increases stability of α B-crystallin (Sun and Liang 1998; Horwitz et al. 1999). Protein interaction also increases solubility; β A2- and β A4-crystallin normally have low solubility but become highly soluble in the presence of β B2- or β B1-crystallin (Marin-Vinader et al. 2006; Liu and Liang 2007). Protein-protein interactions for insoluble proteins are difficult to study with either spectroscopy or a two-hybrid system. In the two-hybrid system assay, the insoluble target protein is unlikely to be able to move to the nucleus for transcription; the observation that T5P γ C-crystallin did not interact with other crystallins might thus arise from insolubility of T5P γ C-crystallin (Fu and Liang 2003). The confocal FRET microscopy used here eliminates this obstacle.

Protein aggregates of T5P γ C-crystallin seen in living cells also eliminate the possibility that expressed proteins become insoluble after cell lysis. Many proteins are soluble in cells but become less soluble after being subjected to many purification processes. The observation that T5P γ C-crystallin can be rescued from insolubility by interaction with α A-crystallin poses the intriguing question of why T5P γ C-crystallin causes congenital cataract. The answer lies with the location of γ -crystallin expression. γ -Crystallins are expressed early in lens development and are distributed in the nuclear and inner cortical regions. On closer inspection, the pattern of a Coppock-like cataract induced by T5P γ C-crystallin mutation (Heon et al. 1999) is characterized by opacity in the nucleus, in which γ -crystallins predominate. The magnified,

high-resolution slit-lamp photo of the cataract (Figure 1 in ref 22) shows severe dust and clump opacification of the supranuclear (zonular) region around 360 degrees. Besides a small dust-like opacification of the fetal nucleus, a considerable opacification in the deep cortex of the lens is seen, a sign of serious disturbance of both protein structure and function. We believe that this is caused by a decreased normal protein-protein interaction for T5P mutant that leads to aggregation. Another possibility is that there is insufficient α -crystallin in this region to rescue the T5P γ C-crystallin mutant from insolubilization.

It is not known whether interaction between T5P γ C- and α A-crystallin is due to chaperone function or another type of interaction, as detected by the two-hybrid system (Fu and Liang 2002b) and other methods (Mach et al. 1990; Liang 2004; Ponce and Takemoto 2005). It is reasonable to assume that chaperone interaction is involved, since T5P γ C-crystallin is partially unfolded (Fu and Liang 2002a), but it may not be the predominant mechanism. Otherwise, the pair α A-T5P γ C should have a greater nFRET than the pair α A γ CWT, as shown in Fig. 4. The interaction between α A- and WT γ C-crystallin cannot be due to chaperone function, because the WT γ C-crystallin is unmodified and not partially unfolded. Our results clearly show that the T5P mutation of γ C-crystallin decreases protein-protein interaction, possibly through the T5P mutation's disruption of the interaction sites. We have observed a similar decrease of nFRET in the other congenital cataract caused by R120G mutation of α B-crystallin (Liu et al. 2007). The nature of interactions among crystallins is not known; it is weak and cannot be detected by conventional liquid chromatography. It also needs to be noted that this weak interaction is different from those responsible for dimerization or oligomerization in either α - or β -crystallin, which arises through interactions between β -strands or β -sheets (Koteiche and McHaourab 1999; Liu and Liang 2006). Besides our use of a two-hybrid system and confocal FRET methods, other methods also report similar weak interactions (Mach et al. 1990; Ponce and Takemoto 2005). The significance of this weak interaction is that it will be greatly enhanced in a concentrated solution, such as in the lens, due to exclusion volume effects.

T5P γ C-crystallin is prone to become aggregated and insoluble; the low solubility (~ 0.2 mg/mL) in solution was reported (Fu and Liang 2002a). Like heat-induced unfolding and aggregation, the mechanism underlying the tendency to aggregate is poorly understood. Such protein aggregation is usually associated with neurodegenerative diseases. This kind of aggregation is not to be confused with the protein-protein interaction that is responsible for protein stability and solubility. Thus, we observe the lower protein-protein interaction for T5P γ C-crystallin than WT γ C-crystallin, but greater aggregation for T5P γ C-crystallin than WT γ C-crystallin. The normal protein-protein interaction and aggregation can be easily differentiated in the confocal cell FRET images; the homogeneous regions are due to protein-protein interactions and the densely bright spots are due to protein aggregation. Fluorescence intensities of these spots are oversaturated and cannot be used for FRET analysis.

One may argue that expression of γ -crystallin in HeLa cells is nonlenticular physiology. However, the epithelial cells are similar to HeLa cells in that neither expresses γ -crystallin. Our use of HeLa cells should not impede our observations and interpretation, since we merely used them as hosting media for the detection of protein interaction and not for studying protein or cell function.

In conclusion, our studies indicate that the T5P γ C-crystallin mutation causes protein aggregation and a decrease in protein-protein interaction in the soluble cell cytoplasm and that both can be visualized in living cells by confocal fluorescence. These phenomena may contribute to lens opacity in the congenital cataract.

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Fig. 1. Representative confocal laser scattering microscopy images of HeLa cells transfected with (A) T5P and (B) WT γ C-crystallin. Constructs were transfected to HeLa cells. After culture, laser scanning microscope images were taken. The data show that cells transfected with T5P γ C-crystallin were more likely to form protein aggregates than cells transfected with WT γ C-crystallin.

Fig. 2.

The percentage of cells with protein aggregates cotransfected with various pairs of genes. The x-axis labels are either for transfection with individual genes or cotransfection with pairs of genes. Transfection of T5P γ C-crystallin mutant increases the number cells with protein aggregates about four-fold (*, $p < 0.05$). Cotransfection with α A-crystallin decreases cells with protein aggregates significantly for T5P γ C-crystallin mutant (**, $p < 0.001$). The mean and standard deviation are shown as percentage $\pm$ SD and represent an average of three independent experiments. For each experiment, cells were counted in 30–50 confocal LSM images.

Fig. 3.

Representative laser scanning microscope FRET images: The following donor-acceptor pairs of GFP-RFP were cotransfected: $\gamma C-\gamma C$, $\gamma C-T5P\gamma C$, $T5P\gamma C-T5P\gamma C$, $\alpha A-\gamma C$, and $\alpha A-T5P\gamma C$. Paired constructs were cotransfected to HeLa cells. After culture, laser scanning microscope FRET images were taken from each of the three channels with the following excitation and emission wavelengths: GFP (475/505 nm), RFP (557/585 nm), and FRET (475/585 nm). On top of the columns are labels for the images from the three channels (green, red, FRET), nFRET, and nFRET in pseudocolor. On left panel are labels for the pairs of GFP-RFP. The color bar represents relative degree of net FRET shown in the pseudocolor images.

Fig. 4.

The quantitative normalized FRET values were calculated from laser scanning microscope FRET images. The nFRET values are the average of regions of interest (ROIs) ($n = 50-80$) in three transfection experiments. ROIs were chosen in the areas that contain no protein aggregates. Significant differences were observed among the three pairs $\gamma C-\gamma C$, $\gamma C-T5P$, and $T5P-T5P$ (*, $p < 0.001$), and between the pairs of either $\gamma C-\gamma C$ and $\alpha A-\gamma C$ (#, $p < 0.001$) or $T5P-T5P$ and $\alpha A-T5P$ (**, $p < 0.0001$). The difference between the pair $\alpha A-\gamma C$ and $\alpha A-T5P$ is also significant (##, $p < 0.001$).

Table 1

The forward and reverse primers for subcloning experiments of various crystallins to pAcGFP-C1 and pDsRED-monomer-C1 vectors. The underlined sequences are for *xhoI* and *EcoRI* restriction sites of forward and reverse primers, respectively.

Forward primers	Reverse primers
α A GACCCTCGAGCTGACGTGACCATCCAG	AGCCTAGAATTCTTAGGACGAGGGAGC
γ C TAGCCTCGAGCTGGGAAGATCACCTTC	CGCCGCGAATTCTTAATACAAATCCAC
T5P γ C TATCCTCGAGCTGGGAAGATCCCCTTC	GGTGAATTCCTAGTAGCCAACCCACGT