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Research paper

Characterization of the βγ-crystallin domains of βγ-CAT, a non-lens βγ-crystallin and trefoil factor complex, from the skin of the toad *Bombina maxima*

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ABSTRACT

βγ-CAT is a naturally existing 72-kDa complex of a non-lens βγ-crystallin (α-subunit, CAT-α) and a trefoil factor (β-subunit, CAT-β) that contains a non-covalently linked form of $\alpha\beta_2$ and was isolated from the skin secretions of the toad *Bombina maxima*. The N-terminal region of CAT-α (CAT-αN, residues 1–170) contains two βγ-crystallin domains while the C-terminal region (CAT-αC) has sequence homology to the membrane insertion domain of the *Clostridium perfringens* epsilon toxin. To examine the biochemical characteristics of the βγ-crystallin domains of βγ-CAT, CAT-αN, CAT-αC and CAT-β were expressed in *Escherichia coli*. Co-immunoprecipitation of the naturally assembled βγ-CAT confirmed that the CAT-α and CAT-β complex always exists. Furthermore, recombinant CAT-β bound recombinant CAT-αN. Ca^{2+}-binding motifs were identified in CAT-αN, and recombinant CAT-αN was able to bind the calcium probe terbium. However, the conformation of CAT-αN was not significantly altered upon Ca^{2+} binding. βγ-CAT possesses strong hemolytic activity toward human erythrocytes, and treatment of erythrocytes with βγ-CAT resulted in a rapid Ca^{2+} influx, eventually leading to hemolysis. However, in the absence of extracellular Ca^{2+}, no significant hemolysis was detected, even though the binding and oligomerization of βγ-CAT in the erythrocyte membrane was observed. Our data demonstrate the binding of CAT-β (a trefoil factor) to CAT-αN (βγ-crystallin domains) and provide a basis for the formation of a βγ-crystallin and trefoil factor complex *in vivo*. Furthermore, the βγ-crystallin domains of βγ-CAT are able to bind Ca^{2+}, and βγ-CAT-induced hemolysis is Ca^{2+} dependent.

1. Introduction

In vertebrates, crystallins are structural proteins that define the refractive index and optical properties of the lens tissue. There are three ubiquitous crystallins (α, β and γ); the α-crystallins are related to the small heat-shock proteins, while the β- and γ-crystallins belong to the same superfamily [1]. Proteins in the βγ-crystallin superfamily contain 40-residue repeats of a characteristic Greek key motif, and two motifs associate with pseudosymmetry to form one domain [2]. EP37 proteins and the mammalian absent in melanoma 1 (AIM1) are non-lens βγ-crystallins that have been described in vertebrates. EP37 proteins are found in the embryonic epidermis, cutaneous glands and gastric epithelial cells of the amphibian *Cynops pyrrhogaster* [3,4], and AIM1 mRNAs of different transcript sizes are temporally regulated during embryogenesis and found in adult skin, heart, lung and liver [5,6]. Although a role in epidermal development and tumor suppression has been proposed for both the EP37 proteins and AIM1 [3–6], little is known regarding the biochemical properties, functions and mechanisms of these non-lens βγ-crystallins in vertebrates.

Some βγ-crystallin superfamily proteins have been shown to bind Ca^{2+} [7–13]. For example, microbial βγ-crystallin member protein S is able to oligomerize to form a multilayer protective coat under stress conditions in a Ca^{2+}-dependent manner [11]. *Yersinia* crystallin is intrinsically unstructured in the apo form and forms β-sheets upon Ca^{2+} binding [12]. AIM1-g1 (the first βγ-crystallin domain of AIM1) has also been shown to bind Ca^{2+} [13,14].

Abbreviations: AIM1, absent in melanoma 1; MBP, maltose-binding protein; CD, circular dichroism; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-1-thiogalactopyranoside; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; IP, immunoprecipitation; FITC, fluorescein 5(6)-isothiocyanate; BSA, bovine serum albumin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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In a previous report, we purified a protein complex (termed βγ-CAT) with a native molecular weight of 72 kDa from the skin secretions of the toad *Bombina maxima*. βγ-CAT is highly active and has multiple effects on mammalian cells, including inducing hemolysins of human erythrocyte via the formation of oligomers in the erythrocyte membrane as well as promoting cell migration, cell detachment and apoptosis, all of which are dependent on the dosage used [15–17]. βγ-CAT is toxic to mammals and induces hypotension and cardiac arrest [18]. It elicits an endothelium-dependent myocardial depression on isolated rabbit hearts, which mimics acute heart failure [19]. βγ-CAT contains two protein components, a 38-kDa chain (CAT-α) and a 18 kDa chain (CAT-β, which consists of three trefoil factor domains) [15]. CAT-α and CAT-β exhibit significant sequence homology with vertebrate non-lens βγ-crystallins, such as human AIM1 and *C. pyrrhogaster* EP37 proteins, and mammalian trefoil factors, respectively [15]. CAT-α actually consists of two distinct parts: the N-terminal region (CAT-αN, residues 1–170) contains two βγ-crystallin domains, and the C-terminal region (residues 173–287) has homology with an internal fragment (residues 118–209) of the *Clostridium perfringens* epsilon toxin (ETX) [20], a region that is thought to be important in the insertion of ETX into the membrane.

Previous studies have shown that the two components of βγ-CAT are functionally associated. CAT-β is probably responsible for cell binding, and CAT-α is able to form transmembrane pores in erythrocyte membranes [15]. To elucidate the biological functions and biochemical mechanisms of βγ-CAT, we examined the structure–function relationship of each component of βγ-CAT in detail. We expressed the various parts of βγ-CAT, namely CAT-αN, CAT-αC and CAT-β, in *Escherichia coli* and examined the biochemical characteristics of CAT-αN. In this report, we demonstrate the binding of CAT-αN to CAT-β and the Ca^{2+}-binding capacity of CAT-αN. Finally, we found that the hemolytic activity of βγ-CAT is dependent upon extracellular Ca^{2+}.

2. Materials and methods

2.1. Materials

E. coli strains DH5α and BL21 (DE3) and the expression vector pET-17b(+) were purchased from Novagen (Darmstadt, Germany). The expression vector pMAL-c2X and the amylose resin were purchased from New England Biolabs (Beverly, MA). Restriction enzymes, PCR polymerase and the simple vector pMD-19T were purchased from TaKaRa (Dalian, China). T4 DNA ligase, the plasmid mini-prep kit and the gel extraction kit were purchased from Tiangen Biotech (Beijing, China). Protein A agarose was purchased from Invitrogen (Manassas, VA, USA). Terbium chloride, fluo-3-AM and all other reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated. Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). βγ-CAT purified from the skin secretions of *B. maxima* and the anti-βγ-CAT antiserum were prepared as previously described [15].

2.2. Cloning, expression and purification of CAT-αN, CAT-αC and CAT-β

The coding sequences of CAT-αN and CAT-αC were amplified from the cDNA of CAT-α using the polymerase chain reaction (PCR). CAT-αN and CAT-αC were subcloned into pMAL-c2X to generate the MBP-fusion protein expression vectors (MBP-CAT-αN and MBP-CAT-αC). CAT-β was subcloned into pET-17b(+) to generate a C-terminal hexahistidine-tagged protein expression vector (CAT-β-His_6). Clones were verified by DNA sequencing. The expression vectors were transformed into *E. coli* strain BL21 (DE3) pLysS. Transformed cells were grown at 37 °C to an OD_{600} of 0.6 and then induced with 1 mM IPTG for 4 h at 37 °C. After centrifugation, the harvested cells were lysed by sonication. The recombinant proteins were purified using nickel chelate or amylose affinity chromatography columns according to the manufacturer's instructions and further purified by ion-exchange chromatography.

2.3. Preparation of rabbit polyclonal antibodies against MBP, MBP-CAT-αN, MBP-CAT-αC and CAT-β-His_6

The specific antibodies against MBP, MBP-CAT-αN, MBP-CAT-αC and CAT-β-His_6 were prepared as previously described [15]. Each of the fusion proteins was used as an antigen to immunize male New Zealand white rabbits. The polyclonal antibodies (IgG) were purified from harvested rabbit serum by double ammonium sulfate precipitation (35% final concentration) and then dialyzed against PBS. The specificity of the antibodies against each protein was confirmed by ELISA. The negative control IgG was purified from pre-immunized rabbit serum.

2.4. Immunoprecipitation and western blotting

For immunoprecipitation (IP), βγ-CAT purified from *B. maxima* skin secretions was precleared with protein A agarose for 1 h at 4 °C in IP buffer (10 mM Tris–HCl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) and recovered by centrifugation (16,000×*g* for 2 min) [21]. The protein was then incubated with the appropriate antibody for 16 h at 4 °C. After incubation with 10 μl of prewashed protein A agarose for 1 h, the unbound proteins were removed. The immunoprecipitates were then washed five times with IP buffer and resuspended in SDS loading buffer. Samples were separated by 12% SDS-PAGE and electrophoretically transferred to a PVDF membrane. The membranes were blocked with 3% BSA in PBS and then probed with the appropriate primary antibody. The washed blots were incubated with an HRP-conjugated secondary antibody, and the blots were developed using chemiluminescence.

2.5. MBP pull-down assays

Purified CAT-β-His_6 was precleared with amylose resin for 1 h at 4 °C in IP buffer and then incubated with immobilized MBP-fusion proteins bound to amylose resin for 16 h at 4 °C [22]. After five washes with IP buffer, the amylose resin was resuspended in SDS loading buffer, and the precipitated proteins were separated by 12% SDS-PAGE and analyzed using western blotting.

2.6. Sequence alignment and molecular modeling

The amino acid sequence of CAT-αN was aligned with members of the βγ-crystallin superfamily. The structure of CAT-αN was predicted using 3D-Jury meta-predictor. After the most common high scoring protein was identified from the ranked 3D-Jury list, the CAT-αN sequence was threaded onto that three-dimensional structure using the homology modeling program ESyPred3D [23].

2.7. Circular dichroism (CD) spectroscopy

CD spectra were recorded on a Jasco J-810 spectropolarimeter at room temperature. Proteins were dissolved in a metal-free solution (20 mM Tris–HCl, 100 mM KCl, 1 mM DTT, pH 7.5), and the protein concentrations used were 0.125 μM for MBP and 0.121 μM for MBP-CAT-αN. The path length used was 0.02 cm, and CD spectra were recorded in the 200–250 nm region. Secondary structure estimations from the CD spectra were analyzed using Yang's algorithm. Spectra were corrected for buffer absorbance.