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Melanoma cell growth inhibition by βγ-CAT, which is a novel non-lens betagamma-crystallin and trefoil factor complex from frog *Bombina maxima* skin

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ABSTRACT

In vertebrates, non-lens βγ-crystallins are widely expressed in various tissues but their functions are unknown. The molecular mechanisms of trefoil factors, initiators of mucosal healing and being greatly involved in tumorigenesis, have remained elusive. βγ-CAT, which is responsible for the potent hemolytic activity and lethal toxicity on mice of frog *Bombina maxima* skin secretions, is the first example of a non-lens βγ-crystallin and trefoil factor complex. Its α- and β-subunits show significant sequence homology to human Absent In Melanoma 1 and trefoil factors, respectively. Here, we showed that βγ-CAT triggered two types of cellular responses in human melanoma cells. On one hand, the protein (25–200 pM) was able to stimulate cell migration in melanoma A375 cells. On the other hand, it inhibited cell proliferation by delaying S-G2/M cell phase transition. Blockade of the cell cycle was associated with increased p21/WAF1 expression, and reduced amounts of Cdc2 and Cdc25C. βγ-CAT also reduced Cdc2 function by increasing the level of inactivated phospho-Cdc2. In addition, the expression of JunB, a well-known cell proliferation inhibitor, was significantly up-regulated. These results provide the first evidence of an anti-proliferative role for a non-lens βγ-crystallin member and action mechanism via association with a trefoil factor.

1. Introduction

Crystallins are structural proteins that define the refractive index and the optical properties of the lens tissue. α-Crystallins are related to the ubiquitous small heat-shock proteins, while βγ-crystallins belong to the same superfamily, which also includes microbial stress-inducible proteins (Wistow and Piatigorsky, 1988). Proteins in βγ-crystallin superfamily contain repeats of a characteristic Greek key motif of about 40 residues and 2 motifs associate with pseudosymmetry to form one domain. Ep37 proteins found in embryonic epidermis, cutaneous glands and gastric epithelial cells of amphibian *Cynops pyrrhogaster* (Takabatake et al., 1992; Ogawa et al., 1998), and mammalian Absent In Melanoma 1 (AIM1) mRNAs with different transcriptional sizes that are temporally regulated during embryogenesis and also found in adult skin, heart, lung, liver, are non-lens βγ-crystallins described in vertebrates (Ray et al., 1997). Although participation in epidermis differentiation and a tumor suppression function have been proposed for ep37 proteins and AIM1 gene (Takabatake et al., 1992; Ray et al., 1996; Ray et al., 1997; Ogawa et al., 1998; Lehmann et al., 2008), little is known about the cellular functions and action mechanisms of these non-lens βγ-crystallins.

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The trefoil factors (TFFs) are secreted proteins that are characterized by a conserved motif that consists of some 40 amino acid residues known as the trefoil domain (or P-domain previously) (Thim and May, 2005). One of the main functions of these peptides is in mucosal protection and healing by stimulating the migration of cells at the mucosal wounding edges (Taupin and Podolsky, 2003). TFFs have been shown to interfere with crucial biological processes such as cell proliferation, differentiation, apoptosis and angiogenesis, and there is convincing evidence that TFFs do play an important role in tumorigenesis (Lefebvre et al., 1996; Regalo et al., 2005). However, key questions remain to be resolved to achieve a full understanding of the first-hand actions of TFFs and the molecular mechanisms involved. So far, no "receptor" that can mediate the functional effects of TFFs has yet been identified, leading to the speculation that they might not act alone (Taupin and Podolsky, 2003).

In screening of novel biotoxins from amphibian, βγ-CAT, which is responsible for the potent hemolytic activity and lethal toxicity on mice of the skin secretions of Chinese red belly frog (*Bombina maxima*), was identified and characterized to be the first example of a naturally existing complex of a non-lens βγ-crystallin and a trefoil factor (Liu et al., 2008). Its α- and β-subunits, with a non-covalently linked form of $\alpha\beta_2$, show significant sequence homology to human AIM1 (Ray et al., 1997) and TFFs (Tomasetto et al., 1990; Hauser et al., 1993), respectively (Liu et al., 2008). βγ-CAT possessed potent lethal toxicity on mammals resulted from hypotension and cardiac inhibition (Qian et al., 2008). The protein elicited an endothelium-dependent myocardial depression on isolated rabbit hearts, which mimicked acute heart failure (Qian et al., in press). Depending on the dosages used, βγ-CAT has been shown to stimulate cell migration and cell apoptosis in primary cultured human umbilical vein endothelial cells (HUVECs). Rapid endocytosis of βγ-CAT in HUVECs via intracellular vacuole formation and potential translocation to cell nucleus were also observed (Liu et al., 2008). In this study, we investigated the cellular effects of βγ-CAT in human melanoma cells. In addition to stimulation of cell migration in melanoma A375 cells, we observed that the protein potently inhibited the cell proliferation. Furthermore, to establish the anti-proliferation mechanism of βγ-CAT, we assayed the cell cycle control-related molecules, which are strongly associated with the cell proliferation signal transduction pathway. For the first time, our findings revealed a potent anti-proliferative action of a non-lens βγ-crystallin member and a trefoil factor complex.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO BRL (Gaithersburg, MD). Penicillin, streptomycin, dimethyl sulfoxide (DMSO), ribonuclease (RNase), propidium iodide (PI) and Hoechst 33342 were purchased from Sigma Chemical (St. Louis, MO). The antibodies specific to cyclin A, cyclin B1, Cdc2, Cdc25C, phospho-Cdc2, phospho-Cdc25C, and p21/WAF1 were obtained from Cell Signaling Technology (Beverly, MA), and a monoclonal antibody against β-actin was purchased from Abcam (Cambridge, MA). βγ-CAT was purified as described previously (Liu et al., 2008). Protein concentration was determined by a protein assay kit (Bio-Rad, CA) with bovine serum albumin (BSA) as a standard.

2.2. Cell culture

Human melanoma cell line A375 was obtained from The Kunming Cell Bank of Type Culture Collection, The Chinese Academy of Sciences. A375 cells were grown in DMEM medium supplemented with 10% FBS (containing 100 IU/ml penicillin and 100 μg/ml streptomycin).

2.3. Cell migration

Stimulation of cell migration was first tested by a modified Boyden chamber assay according to the instructions of the manufacturer (Chemicon, CA). The migration activity was expressed as the value monitored at 550 nm of extraction. Alternatively, wound healing assay was performed as described previously (Liu et al., 2008). Briefly, confluent A375 cells cultured in six-well collagen type I pre-coated culture plates (Nunc, USA) were scratched by a standard 200-μl pipette tip across the diameter of the wells. After the plates were rinsed twice with starve medium (DMEM containing 0.5% FBS only), fresh starve medium containing βγ-CAT (25–200 pM) or epidermis growth factor (EGF; Invitrogen, USA) (400 pM) was added into the plates, and changed every 24 h. Time-lapse photography of the wounding edges was performed under an inverted-phase microscopy within 72 h. The wound closure in the presence of βγ-CAT was estimated by comparing the wound area at various time points with that of the zero time.

2.4. Cell proliferation assay

The proliferation curve of A375 cells incubated with βγ-CAT was constructed by MTT method (Alley et al., 1988). A375 cells were seeded at a density of 1×10^3 cells/ml in 96-well cell culture plates, which had been grown in serum-free medium for 24 h to enrich the cells in G1 phase. Fresh medium supplemented with 10% FBS containing βγ-CAT (25 pM to 25 nM) was added into the plates and changed every 48 h. The viable cell population was determined by MTT assay for 7 days.

In the parallel experiments, the viability of A375 cells was detected in flow cytometry (Becton Dickinson, Mountain View, CA). Briefly, 3 ml of A375 cells starved for 24 h were seeded at a density of 1.5×10^5 cells/ml in 25 cm^2 flasks and were grown in fresh medium supplemented with 10% FBS containing βγ-CAT (25 pM to 25 nM) for 1–7 days. Every 24 h, the cells were collected by trypsinization. After washing with PBS, the cells were resuspended in 150 μl of PBS containing 5 μg/ml Hoechst 33342, and incubated for 10 min in dark at 4 °C. PBS containing 100 μg/ml PI and 100 μg/ml RNase was added and incubated for another 10 min in dark at 4 °C. Samples were analyzed by flow cytometry and 10,000 events/sample were recorded.