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A novel annexin A2 protein with platelet aggregation-inhibiting activity from amphibian *Bombina maxima* skin

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ABSTRACT

Annexin A2 is a unique member of annexin family with multi-functions in membrane physiology, implicated in inflammation and cancer progression. mRNA of Annexin A2 is abundant in the skin of some amphibians. However, no annexin A2 protein has been isolated and characterized from amphibian skin. In this report, a novel annexin A2 protein with apparent molecular weight of 33 kDa and named Bm-ANXA2, was purified from frog *Bombina maxima* skin, which is highly toxic to mammals, by a combination of ion exchange and gel filtration chromatography. A full-length cDNA encoding the protein was obtained from the cDNA library constructed from the frog skin. Sequence analysis indicates that Bm-ANXA2 shares 89% and 80% amino acid sequence identities with those of *Xenopus* and human annexin A2, respectively. Different from other annexin A2 proteins, the N-terminal 26 amino acids of Bm-ANXA2 were truncated. Bm-ANXA2 dose-dependently inhibited human platelet aggregation stimulated by various agonists in a Ca^{2+}-dependent manner. It bound to activated platelets and significantly inhibited $\alpha_{IIb}\beta_3$ activation and α-granular secretion. This is the first report that an annexin A2 protein possesses platelet aggregation-inhibiting activity, providing novel clues in the illustration of pathophysiological roles of annexin A2 proteins.

1. Introduction

Amphibian skin is a morphologically, physiologically and biochemically complex organ that fulfills a wide range of functions for amphibian survival, such as antimicrobial defense, anti-predator, respiration, water regulation, temperature control, and reproduction (Duellman and Trueb, 1986). Over the past several decades, numerous studies have focused on the bioactive components in amphibian skin. A lot of peptides with diverse biological activities have been isolated and characterized. However, the physiological and functional mechanisms of amphibian skin are incompletely understood (Clarke, 1997).

Annexins are a well-known multigene family of Ca^{2+}-regulated membrane-binding and phospholipid-binding proteins (Rescher and Gerke, 2004). Annexin A2 (ANXA2) (also termed annexin II) is a unique member of annexin family with multi-functions in membrane physiology and cell proliferation and is related to inflammation and cancer progression (Gerke et al., 2005; Yamane et al., 2009). ANXA2s are generally cytosolic proteins without any typical secretory signal peptide, while ANXA2 may be expressed at the cell surface in some certain instances. The ANXA2 knockout mouse has defects in angiogenesis and

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fibrin homeostasis, due most likely to a failure to localize plasmin on the endothelial cell surface (Ling et al., 2004). ANXA2 functions as a co-receptor for tissue plaminogen activator, which is crucial for the degradation of fibrin. The study of human with acute promyelocytic leukemia also suggested that overexpression of ANXA2 on leukemic blast cell surface led to dysregulated plasmin generation and a hyperfibrinolytic hemorrhagic state (Menell et al., 1999). However, how annxins are secreted to the cell surface is one of the long-standing problems remained to be addressed.

Exogenous proteins affect platelet function by binding or degrading platelet receptors, activating protease-activated receptors or modulating ADP release and thromboxane A2 (TXA2) formation (Clemetson et al., 2001). Many of these purified components are valuable tools in platelet research, providing new information on receptor function and signaling. For example, snake venoms contain many components possessing platelet aggregation-inhibiting activity (Clemetson et al., 2007). Disintegrins, containing a RGD or KGD sequence, block integrins including the fibrinogen receptor glycoprotein (GP)IIb/IIIa, the vitronectin receptor ($\alpha v\beta 3$) and the fibronectin receptor ($\alpha 5\beta 1$) (Scarborough et al., 1993). L-amino acid oxidases inhibited platelet aggregation possibly through the continuous generation of H_2O_2 (Lu et al., 2005). However, amphibian skin components affecting platelets have not been well studied.

The Chinese red belly toad, *Bombina maxima*, is an endemic amphibian in the mountainous area of southwestern China. It has long been known by the indigenous people that the toad lives in the very harsh environment and its skin is highly toxic to mammals. Recently, a naturally existing complex of non-lens $\beta\gamma$-crystallin and trefoil factor, named $\beta\gamma$-CAT, was identified from the skin, and its lethal toxicity to rabbits was mainly resulted from endothelium-dependent myocardial depression (Qian et al., 2008a, b). Intravenous injection with $\beta\gamma$-CAT significantly decreased the counts of platelets (Qian et al., 2008a) and $\beta\gamma$-CAT lysed the human platelets in our *in vitro* assay (Zhang et al, unpublished observation). Additionally, a novel two domain trefoil factor, Bm-TFF2, was isolated form the skin secretion and was able to induce human platelet activation (Zhang et al., 2005a). We are interested to look for other components of the frog skin affecting the human platelets. In this study, we present the characterization and molecular cloning of *B. maxima* annexin A2 (Bm-ANXA2) from the skin homogenate. Bm-ANXA2 is the first annexin A2 protein reported to possess platelet aggregation-inhibiting activity.

2. Materials and methods

2.1. Materials

DEAE-Sephadex A-50, Sephadex G-50 (superfine), Mono-S and Fluorescence indictor Cy™3 were from GE Biosciences (Uppsala, Sweden). Fluorescein 5(6)-isothiocyanate (FITC)-coupled anti-CD62P, anti-$\alpha_{IIb}\beta_3$ (PAC-1) and control IgG antibodies were from BD Biosciences (San Jose, CA, USA). Stejnulxin was purified from snake *Trimeresurus stejnegeri* venom as previously described (Lee et al., 2003). All other reagents were from Sigma (St. Louis, MO, USA) unless otherwise indicated. The protein concentration was determined by a protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard.

2.2. Preparation of frog skin homogenate

The preparation method for freeze-dried powder of *B. maxima* skin homogenate was as previously described (Zhang et al., 2005b). Adult specimens of *B. maxima* of both sexes were collected from Chuxiong County, Yunnan Province, Southwest China. The frog was anesthetized with ether, and then the skin was peeled and washed in 50 mM Tris–HCl buffer (pH 7.8), containing 5 mM EDTA and 0.1 M NaCl. The skin was cut into small pieces, and homogenized in the same buffer. After centrifugation, the homogenate supernatant was collected, lyophilized, and stored at −80 °C.

2.3. Preparation of washed platelets, platelet aggregation and inhibition

The methods were mainly described previously (Zhang et al., 2005a). Briefly, human platelet rich plasma was from the KunmingBlood Center. After successive washes by centrifugation, the platelet pellets were resuspended in Tyrode's buffer (137 mM NaCl, 2 mM KCl, 0.3 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.5 mM glucose, 0.35% BSA, pH 7.4) at a concentration of 5×10^8 platelets/ml. Washed platelets were preincubated with or without 2 mM $CaCl_2$ at 37 °C for 5 min before adding the samples. For inhibition study, different concentrations of Bm-ANXA2 were incubated with platelets for 5 min before the agonist was added.

2.4. Platelet-binding activity of Bm-ANXA2

The assay of platelet-binding activity was performed as reported (Wang et al., 2006). Briefly, flat-bottom 24-well plates were coated with different concentrations of Bm-ANXA2 at 4 °C for 12 h. After washed with Tyrode's buffer containing 0.1% Tween 20, the wells were blocked with 2% BSA. Then the washed platelets (5×10^5/ml) in Tyrode's buffer with 2 mM $CaCl_2$, 2 mM EDTA, or 2 mM $CaCl_2$ combined 1 mM RGDS were added. Platelets were activated with ADP at 37 °C for 5 min. After washing three times, the bound platelets were counted under light microscope. Nonspecific binding was measured in the presence of 5 mM EDTA.

2.5. Flow cytometric analysis

For examination of $\alpha_{IIb}\beta_3$ activation or P-selectin expression, washed platelets were activated with an agonist in the presence of FITC-PAC-1 (1:100) or FITC-anti-CD62P (1:100) at 37 °C without stirring. FITC labeled isotype-matched antibodies were used as controls. After incubated for 20 min, the platelets were washed and then analyzed with flow cytometry (FACSVantage SE, BD Biosciences). For detecting stejnulxin binding, washed platelets (5×10^5/ml) with 2 mM $CaCl_2$ were incubated with different concentrations of Bm-ANXA2 for 10 min.