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Letter to the editor Open Access

Amphibian pore-forming protein βγ-CAT drives metabolite release from small extracellular vesicles through channel formation

DEAR EDITOR,

Pore-forming proteins (PFPs) are widely distributed among all kingdoms of life and can oligomerize to form pores/channels in membrane systems. Extracellular vesicles (EVs) circulate in all biological fluids and can trigger biological responses at a distance, thus emerging as an additional means of intercellular communication through the release of cellular cargo, such as lipids, nucleic acids, metabolites, and proteins. To date, however, the mechanism by which EV contents are released into extracellular space remains unclear. In our previous study on toads (*Bombina maxima*), we identified a PFP and trefoil factor complex βγ-CAT (βγ-crystallin fused aerolysin-like protein (α-subunit) and trefoil factor (β-subunit) complex, hence named βγ-CAT), which assembled under strict regulation in response to environmental cues. Here, we observed that the PFP βγ-CAT colocalized with EVs in the skin of *B. maxima in vivo*. Using small EVs (sEVs) isolated from *B. maxima* dermal fibroblast cells and murine fibroblast cells, we found that βγ-CAT could specifically target and oligomerize on purified EVs, rather than disrupt membrane integrity, and promote the release of different metabolites. Our analysis revealed that a secretory PFP drove metabolite release from EVs through channel formation, providing novel clues for the delivery of EV content into extracellular space.

PFPs are non-classical membrane proteins usually secreted in a water-soluble monomeric form with wide distribution in various organisms (Dal Peraro & van der Goot, 2016). Upon reaching their target cell membrane, these proteins can oligomerize to form a transmembrane pore structure of various sizes (1–30 nm), which function as channels for the passage of diverse molecules (Dal Peraro & van der Goot, 2016). For example, gasdermin D, the executor of pyroptosis, can oligomerize to form pores in the cell membrane and facilitate the release of cytoplasmic contents, such as the inflammatory cytokine interleukin-1β (IL-1β), into the extracellular space (Ding et al., 2016). Based on the secondary structure elements that are inserted into the target membrane, PFPs can be characterized as α-helix and β-barrel PFPs (Dal Peraro & van der Goot, 2016; Zhang et al., 2021). Pore-forming toxins (PFTs) represent the largest family of bacterial toxins and are a significant subclass of PFPs. Aerolysin, secreted by the gram-negative bacterium *Aeromonas hydrophila*, is a β-barrel PFT and a major virulence factor for infection (Dal Peraro & van der Goot, 2016; Zhang et al., 2021). Interestingly, proteins adopting a similar structure to that of aerolysin have also been found in other species besides bacteria, termed as aerolysin family PFPs (af-PFPs) or previously as aerolysin-like proteins (ALPs) (Zhang et al., 2021).

Amphibian skin, which contains many bioactive molecules, is responsible for respiration, water balance, metabolism, and immune defense (Zhang, 2015). We previously isolated and identified a protein complex, named βγ-CAT, composed of an af-PFP (βγ-crystallin domain fused with an aerolysin domain, termed BmALP1, α-subunit) and a trefoil factor (BmTFF3, β-subunit) from the skin secretions of the *B. maxima* toad (Zhang et al., 2021).

As a secretory PFP, the βγ-CAT assembly can be negatively regulated by its paralog (aerolysin family PFP BmALP3, which lacks membrane pore formation capacity) in an oxygen-dependent manner, such that it exists in active or inactive form in the extracellular environment. Upon activation, βγ-CAT targets acidic glycosphingolipids in lipid rafts via a double-receptor binding model, leading to the endocytosis of its α-subunit BmALP1, which stimulates pinocytosis, resulting in the formation of a channel along the cellular endolysosomal pathway. This action changes the biochemical contents and properties of intracellular vesicles and modulates cell exocytosis, including the release of functional EVs, depending on cell conditions (Zhang et al., 2021). Thus, βγ-CAT and its regulatory network form a secretory endolysosomal channel (SELC) pathway, representing a novel PFP-driven cell vesicular delivery system (Zhang et al., 2021). In toads, based on cellular endocytosis and exocytosis pathways, the SELC protein βγ-CAT plays multiple roles in environmental adaptation, including immune defense, tissue repair, water maintenance under osmotic stress, and nutrient acquisition and delivery during starvation (Liu et al., 2023; Shi et al., 2022; Zhang et al., 2021).

EVs are membrane-enclosed structures secreted by various types of cells and found in almost all biological fluids (van Niel et al., 2018). They can be classified as microvesicles (MVs) or

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exosomes based on their mode of release and size. MVs range from 30 to 1 000 nm in diameter and are released through the outward budding of the plasma membrane, whereas smaller exosomes range from 30 to 150 nm and are secreted by the fusion of outer multivesicular bodies (MVBs) to the plasma membrane (van Niel et al., 2018). EVs carry a cargo of bioactive molecules, including proteins, nucleic acids, lipids, and metabolites, which can be delivered to recipient cells through receptor-mediated endocytosis, macropinocytosis, or membrane fusion (Maas et al., 2017). While certain molecules contained in EVs, such as cytokines, growth factors, and metabolites, can act on cell surface targets or demonstrate direct biological effects in extracellular environments, the specific mechanisms by which these molecules are released into the extracellular space remain unclear (Maas et al., 2017; Zhang et al., 2021).

Given the membrane structure of EVs and channel formation characteristics of βγ-CAT, which promote vesicle transport, we hypothesized that exposure to βγ-CAT may induce the release of EV contents.

βγ-CAT, a major component in *B. maxima* skin secretions, is distributed in the epidermis, dermis, and glands (Zhang et al., 2021). We first evaluated the localization of βγ-CAT and EVs in *B. maxima* skin through immunofluorescence (IF) assay to establish the physiological significance of βγ-CAT and EVs *in vivo*. Our results indicated that βγ-CAT was present in various regions of the toad skin (Figure 1A), similar to our previous study (Zhang et al., 2021). Similarly, EV marker CD63 displayed broad distribution in the toad skin and strong colocalization with βγ-CAT (Figure 1A). Furthermore, immunoelectron microscopy (IEM) confirmed the localization of βγ-CAT in EVs in toad skin (Figure 1B). These findings indicate that βγ-CAT is colocalized with EVs *in vivo*, suggesting that βγ-CAT may be involved in the functions of EVs.

To further investigate the direct effects of βγ-CAT on EVs, we cultured and confirmed a dermal fibroblast cell line derived from the skin of *B. maxima* tadpoles (BMDF) established in our previous study (Xiang et al., 2012). Karyotype analysis showed that the chromosome number of the cultured BMDFs was 2*n*=28, including six pairs of large chromosomes, one pair of medium chromosomes, and seven pairs of small chromosomes (Supplementary Figure S1A). Moreover, the expression of the fibroblast biomarker vimentin was also detected in the cultured BMDF lysates by western blotting (Supplementary Figure S1B), consistent with our previous study (Xiang et al., 2012). In addition, the mouse L929 fibroblast cell line was cultured and used in subsequent study.

The cultured BMDF and L929 supernatants were collected to isolate sEVs by differential centrifugation (Supplementary Figure S2). The purified BMDF- and L929-derived sEVs were then characterized by size exclusion using nanoparticle tracking analysis (NTA), revealing a polydisperse particle population ranging from 30 to 300 nm in diameter with a modal diameter of 150 nm (Figure 1C). Western blot analysis showed that the sEVs from the BMDMs and L929 fibroblasts were both positive for EV-specific biomarkers CD63, CD81, and Tsg101 (Figure 1D). The morphology of isolated BMDF- and L929-derived sEVs was analyzed by transmission electron microscopy (TEM), which showed that sEVs isolated from the two cell lines were spherical (Figure 1E), a classical EV morphology. These results confirmed the successful isolation of BMDF-sEVs and L920-sEVs from the cultured cell medium.

After isolating the BMDF-sEVs and L929-sEVs, we next explored the binding ability of βγ-CAT to sEVs *in vitro*. We used 10 nmol/L βγ-CAT, which is within the physiological concentration range in *B. maxima* (Zhang et al., 2021), for subsequent experiments. Western blotting showed that βγ-CAT formed obvious oligomers on the purified BMDF-sEVs and L929-sEVs (Figure 1F). Oligomerization on the membrane is considered a key step for PFP pore formation (Dal Peraro & van der Goot, 2016). Using a liposome model, we also recently found that oligomerized βγ-CAT can form pores (Liu et al., 2021). These findings indicate the occurrence of

βγ-CAT oligomerization on the purified sEVs, suggesting that βγ-CAT may form pores on sEVs.

To further explore the effect of βγ-CAT on sEV membrane integrity, we observed sEVs treated with βγ-CAT using TEM. In our previous study, we found that LL-37, an antimicrobial peptide in humans, can lyse viral envelopes (Liu et al., 2021). Thus, LL-37 was used as a positive control in the present study. We found that LL-37 also lysed the sEV membrane (Figure 1G). However, βγ-CAT treatment did not affect sEV membrane integrity, showing similar results to phosphate-buffered saline (PBS) treatment (Figure 1G). These findings suggest that βγ-CAT can form pores on sEVs without disrupting membrane integrity.

After observing that βγ-CAT may form pores/channels on sEVs without lysing the membrane, we investigated whether these channels could release contents into external environments. To address this question, the supernatants from BMDF-EVs treated with βγ-CAT or PBS were collected through a 100 kDa filtering step. In our previous study, we determined that the pores formed by βγ-CAT are approximately 2 nm in diameter (Liu et al., 2021), consistent with the pores of af-PFPs reported so far (Dal Peraro & van der Goot, 2016). Therefore, we explored the metabolites in the βγ-CAT- and PBS-treated sEV supernatants using untargeted metabolomic profiling against a library of more than 3 000 biochemical compounds. Principal component analysis (PCA) was performed to determine the aggregation and description of the samples (Supplementary Figure S3). Notably, the βγ-CAT-treated sEV supernatants were enriched in 92 metabolites (Figure 1H; Supplementary Table S1).

To better understand the metabolites released from the sEVs driven by βγ-CAT pore formation, we subjected the 92 enriched metabolites to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Results showed that the metabolites were enriched in five KEGG pathways, including “purine metabolism”, “pantothenate and CoA biosynthesis”, “lysine degradation”, “arginine and proline metabolism”, and “pyrimidine metabolism” (Figure 1I).

In conclusion, our study demonstrated the direct oligomerization and channel formation of PFP βγ-CAT on sEVs, leading to the release of metabolites into the extracellular space. These findings provide further evidence to support our previous hypothesis that βγ-CAT directly acts on EVs and functions as a secretory EV channel (Zhang et al., 2021). The detailed effects of metabolites released from sEV channels formed by βγ-CAT on *B. maxima in vitro* and *in vivo* will be a fascinating subject for future research.

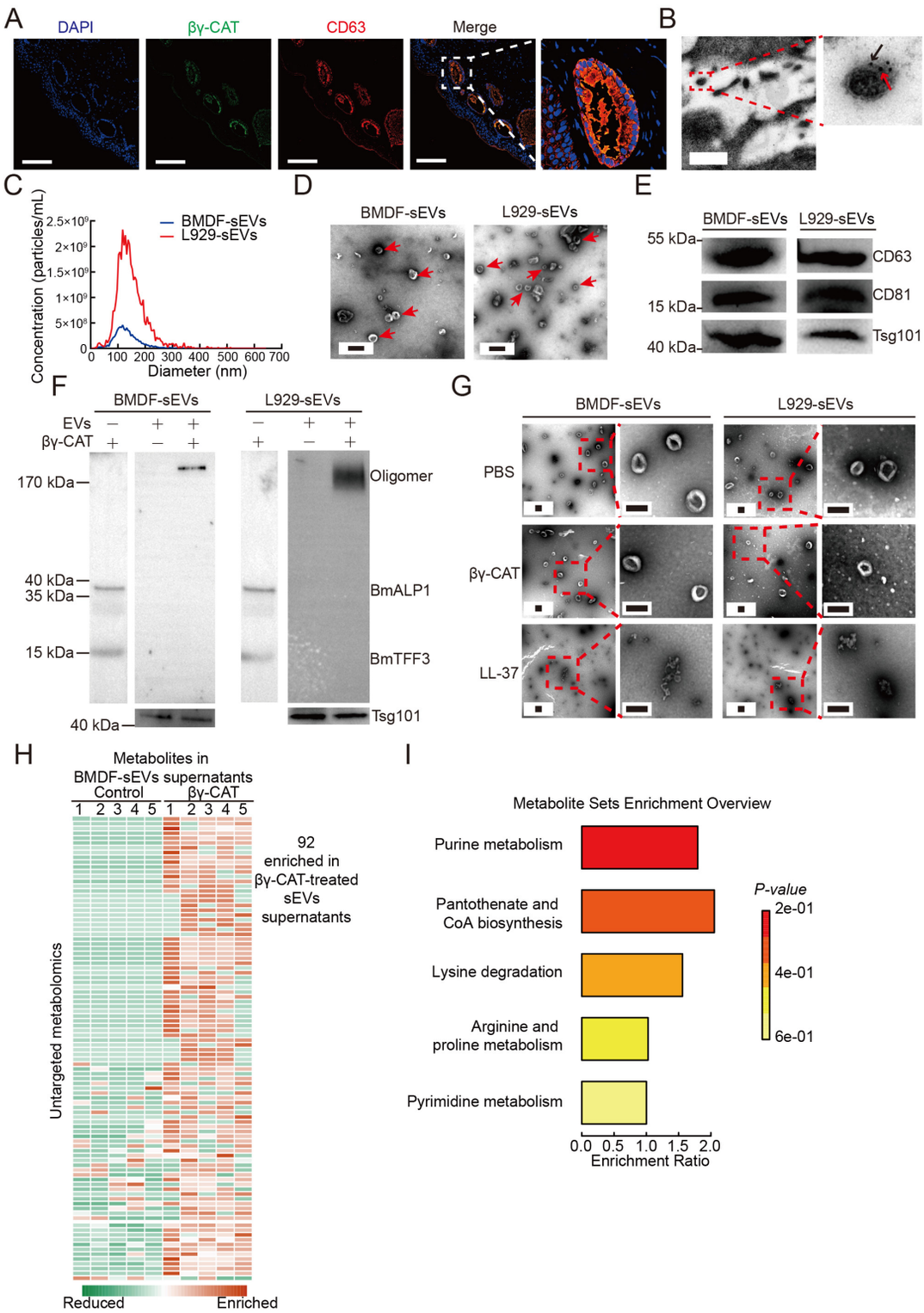

Figure 1 Pore-forming protein βγ-CAT drives metabolite release from sEVs through channel formation

A: Colocalizations of βγ-CAT (green) and EV marker CD63 (red) in toad skin tissues were observed by laser confocal microscopy. Scale bar: 25 μm. B: Colocalizations of βγ-CAT (labeled with 5 nm colloid golden, black arrow) and EV marker CD63 (labeled with 10 nm colloid golden, red arrow) in toad skin were observed by TEM. Scale bar: 200 nm. C: Particle size distribution of sEVs isolated from BMDF (BMDF-sEVs) and L929 (L929-sEVs) cell culture medium by NTA. D: Morphological analysis of purified BMDF-sEVs and L929-sEVs by TEM. Arrows indicate EVs. Scale bar: 200 nm. E: Western blotting of purified BMDF-sEVs and L929-sEVs using EV-specific markers. F: Purified BMDF-sEVs (left) and L929-sEVs (right) were treated with βγ-CAT for 30 min, with appearance of βγ-CAT oligomers on treated EVs then determined by western blotting. G: Purified BMDF-sEVs (left) and L929-sEVs (right) were treated with PBS, LL-37, or βγ-CAT for 30 min, with ultra-morphology of EVs then observed by TEM. Scale bar: 200 nm. H: Heatmap from untargeted metabolomics of supernatants from βγ-CAT-treated and PBS-treated (control) BMDF-sEVs. Data are representative of five biological replicates. Data are related to Supplementary Table S1. I: KEGG pathway enrichment analysis of metabolites enriched in βγ-CAT-treated BMDF-sEV supernatants.

SUPPLEMNTARY DATA

Supplementry data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Q.Q.W., X.Q.L., Y.Z., and Y.X. conceptualized, drafted, and revised the manuscript; Q.Q.W., X.S.W., S.M.X., L.Z.L., X.Q.L., L.Z., X.L.B., Y.Q.G., and X.L.G. performed and analyzed the experiments; Q.Q.W., W.H.L., and Y.Z. analyzed the data. All authors read and approved the final version of the manuscript.

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