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RESEARCH HIGHLIGHT

# V-cGAPs: attenuators of 3′3′-cGAMP signaling

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**Cyclic GMP-AMPs (cGAMPs) are new members of the cyclic dinucleotide family of second messenger signaling molecules identified in both bacteria and mammalian cells. A recent study by Gao *et al.* published in *Cell Research* has identified and characterized three 3′3′-cGAMP-specific phosphodiesterases (termed as V-cGAP1/2/3) in *V. cholerae*, thereby providing mechanistic insights into the function of these enzymes that degrade cGAMPs.**

Despite their indispensable roles in the composition of DNA and RNA, as well as serving as energy sources, nucleotides are also well known as crucial signaling molecules in all domains of life. Cyclic dinucleotides (CDNs) represent an important and growing family of second messengers, which have been previously recognized as key modulators governing a variety of cellular activities in bacteria, and more recently, in mammalian cells. c-di-GMP and c-di-AMP, the first two members of the CDN family, have been implicated in central bacterial processes, and likely act as universal bacterial secondary messengers [1, 2]. The latest addition to the bacterial CDN family is 3′3′-cGAMP, a hybrid molecule that is synthesized from ATP and GTP by DncV (a cyclase from *V. cholera*e) and shown to promote intestinal colonization of *V. cholera*e by downregulating chemotaxis [3]. Predicted homologs of DncV are present in many other bacterial species [3], indicating that 3′3′-cGAMP may also regulate a wide range of cellular functions, similar to c-di-GMP and c-di-AMP. The research on CDNs as second messengers reached new heights following the recent identification of 2′3′-cGAMP, a noncanonical CDN in mammalian cells containing mixed 2′,5′ (at GpA step) and 3′,5′ (at ApG step) linkages, which is synthesized by cGAMP synthase (cGAS) in response to the presence of DNA in the cytosol [4-6]. A remarkable set of new discoveries have revealed that all the CDNs described above are able to bind and activate STING, the central adaptor in the cytosolic DNA sensing pathway, thereby promoting the innate immune response in mammalian cells by inducing the expression of Type I interferon (IFN) [7-9].

Given their critical roles in a variety of important cellular processes, the cellular levels of CDNs have to be tightly controlled by the coordinated action of counteracting cyclases and degradation enzymes. To date, several phosphodiesterases (PDEs) have been found to hydrolyze c-di-GMP (EAL or HD-GYP domain-containing enzymes) [1] and c-di-AMP (DHH-DHHA or HD domain-containing enzymes) [2, 10] (Figure 1). In addition, recent research reported that ENPP1 (ecto-nucleotide pyrophosphatase/phosphodiesterase) is the dominant 2′3′-cGAMP hydrolyzing enzyme in mammalian cells [11] (Figure 1). A new study by Gao *et al*. [12] has now identified the first three 3′3′-cGAMP-specific PDEs in *V. cholera*e and provided detailed insights into their enzymatic mechanisms.

There are a total of 36 potential PDE genes (containing EAL, HD-GYP or DHH domains) in the *V. cholera*e genome. To search for 3′3′-cGAMP-specific PDE(s), Gao *et al*. [12] established an efficient and sensitive eukaryotic screening system by taking advantage of the ability of 3′3′-cGAMP to activate STING and induce type I IFN expression in mammalian cells. By overexpressing the 3′3′-cGAMP synthetase DncV together with the 36 potential PDEs in 293 cells, the authors could monitor IFN-β promoter activation to identify the PDE(s) that could degrade 3′3′-cGAMP. To exclude false-positives, Gao *et al*. further purified the PDEs that potentially target 3′3′-cGAMP based on the initial screening, and incubated these enzymes with chemically synthesized 3′3′-cGAMP. The treated 3′3′-cGAMP molecules were further assayed by either adding to PFO-permeabilized THP-1 cells to examine IRF3 phosphorylation levels or through loading on HPLC to monitor the generation of new products. As a result of the screening and validation, the authors successfully identified three HD-GYP domain-containing proteins that could degrade 3′3′-cGAMP, named VCA0681, VCA0210 and VCA0931 (designated as V-cGAP1, 2 and 3, respectively).

To determine the substrate specificity of V-cGAPs, different cGAMP linkage isomers (3′3′-, 3′2′-, 2′3′-, and 2′2′-cGAMPs) were incubated with the purified V-cGAPs. The results of both IRF3 phosphorylation in THP-1 cells and HPLC assays clearly indicated that V-cGAPs only degrade 3′3′-cGAMP, but not other cGAMP linkage isomers. The 3′3′-cGAMP PDE activity of V-cGAPs was further confirmed by dosage- and time-dependent enzymatic assays. By using mutant proteins, the authors also confirmed that both the HD and GYP motifs within V-cGAPs

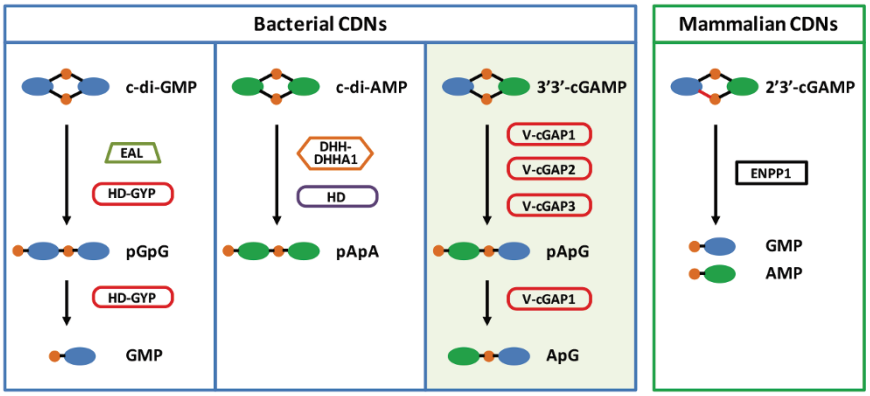


**Figure 1** Schematic representation of degradation enzymes identified for different cyclic dinucleotides and the related hydrolysis products. The various protein domains are highlighted by different shapes and colors. Note that the newly identified V-cGAPs belong to the HD-GYP domain-containing PDEs.

are critical for PDE activity.

Combining detailed HPLC analysis, mass spectrometry and enzymatic treatment, Gao *et al*. definitively established that 3′3′-cGAMP is first hydrolyzed by all three V-cGAPs to generate linear 5′-pApG, which is further hydrolyzed into 5′-ApG only by V-cGAP1. These results show that V-cGAP2 and V-cGAP3 have only PDE activity, while V-cGAP1 has both PDE and 5′-nucleotidase activities. The authors also found that V-cGAP1 has a much higher activity for linearization of 3′3′-cGAMP to 5′-pApG than V-cGAP2 and 3, with the later two V-cGAPs exhibiting similar kinetics of degradation.

The cellular level of 3′3′-cGAMP has to be tightly regulated by a combination of counteracting synthesis and degradation enzymes. Since the expression level of DncV was found to be inducible by outside signals to enhance intestinal colonization and infectivity, it is very likely that the expression level of V-cGAPs will also be regulated by 3′3′-cGAMP production. Indeed, the authors proved that V-cGAP expression is greatly and readily enhanced after arabinose-induced DncV expression in a Δ*dncV* mutant *V. cholera*e strain, at both mRNA (by qRT-PCR) and protein (by immunoblot analysis) levels. To confirm the *in vivo* function of V-cGAPs, the authors performed both "chemotactic" and "infant mouse colonization competition" assays by using V-cGAP1/2/3 single-, double-, or triple-deletion *V. cholera*e strains. All the *in vivo* data clearly established that V-cGAPs counteract DncV function and exert a crucial role in regulating bacterial infectivity.

The large amount of insightful data presented by Gao *et al*. has elucidated detailed information regarding the identification and characterization of 3′3′-cGAMP-specific phosphodiesterases, thereby providing valuable insights into our understanding of the regulatory mechanisms of cGAMP signaling in bacteria. Clearly, further structural work will be necessary to understand the intermolecular interactions between 3′3′-cGAMP and V-cGAPs, and provide insights into the mechanism by which V-cGAPs preferentially attack the phosphodiester bond at the GpA step.

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