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The Innate Immune DNA Sensor cGAS Produces a Noncanonical Cyclic Dinucleotide that Activates Human STING

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SUMMARY

The presence of foreign DNA in the cytosol of mammalian cells elicits a potent antiviral interferon response. Recently, cytosolic DNA was proposed to induce the synthesis of cyclic GMP-AMP (cGAMP) upon binding to an enzyme called cGAMP synthase (cGAS). cGAMP activates an interferon response by binding to a downstream receptor called STING. Here, we identify natural variants of human STING (hSTING) that are poorly responsive to cGAMP yet, unexpectedly, are normally responsive to DNA and cGAS signaling. We explain this paradox by demonstrating that the cGAS product is actually a noncanonical cyclic dinucleotide, cyclic [G(2′-5′)pA(3′-5′)p], which contains a single 2′-5′ phosphodiester bond. Cyclic [G(2′-5′)pA(3′-5′)p] potently activates diverse hSTING receptors and, therefore, may be a useful adjuvant or immunotherapeutic. Our results indicate that hSTING variants have evolved to distinguish conventional (3′-5′) cyclic dinucleotides, known to be produced mainly by bacteria, from the noncanonical cyclic dinucleotide produced by mammalian cGAS.

INTRODUCTION

Recognition of pathogen-derived nucleic acid is a major mechanism by which innate immune responses are initiated in mammals (Barbalat et al., 2011). Several families of germ-line-encoded nucleic acid sensors have been described, including the Toll-like receptors and RIG-I-like receptors (Palm and Medzhitov, 2009; Takeuchi and Akira, 2010). Upon binding nucleic acids, these sensors initiate signaling cascades that lead to the production of cytokines and other immune effector proteins that provide host defense.

The cytosolic presence of foreign double-stranded DNA (dsDNA) triggers a potent antiviral response dominated by the production of type I interferons (IFNs) (Ishii et al., 2006; Stetson and Medzhitov, 2006). An endoplasmic-reticulum-resident host protein called stimulator of IFN genes (STING; also called TMEM173, MITA, ERIS, and MPYS) was shown to be required for IFN response to cytosolic dsDNA (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Sun et al., 2009; Zhong et al., 2008). Bacterially derived second messenger molecules called cyclic dinucleotides (CDNs) can also induce an IFN response (McWhirter et al., 2009) that depends on STING (Jin et al., 2011a; Sauer et al., 2011). CDNs are secreted or released into the cytosol by certain bacterial pathogens (Barker et al., 2013; Woodward et al., 2010) and bind directly to STING (Burdette et al., 2011). Previously, we identified a mutant allele of mouse STING (mSTING), encoding an alanine in place of arginine 231 (R231A), that abolished responsiveness to CDNs but did not appreciably affect the IFN response to cytosolic dsDNA (Burdette et al., 2011). Thus, although the IFN responses to both cytosolic CDNs and dsDNA require STING, the responses to these chemically distinct ligands can be genetically uncoupled.

Two recent papers (Sun et al., 2013; Wu et al., 2013) unified our understanding of the cytosolic response to CDNs and DNA by proposing that the cytosolic presence of dsDNA leads to the production of a CDN, cyclic GMP-AMP (cGAMP), by a DNA-dependent sensor enzyme called cGAMP synthase (cGAS). cGAMP was shown to bind and activate STING, but it remained unclear how the mSTING R231A mutant could still initiate responses to dsDNA while lacking responsiveness to CDNs. Therefore, we sought to investigate the mechanism by which cGAS activates STING.

RESULTS AND DISCUSSION

Our previous work (Burdette et al., 2011; Sauer et al., 2011) focused primarily on mSTING, and it is not yet clear whether all human STING (hSTING) variants can respond to CDNs (Conlon et al., 2013; Jin et al., 2011b). In agreement with previous reports

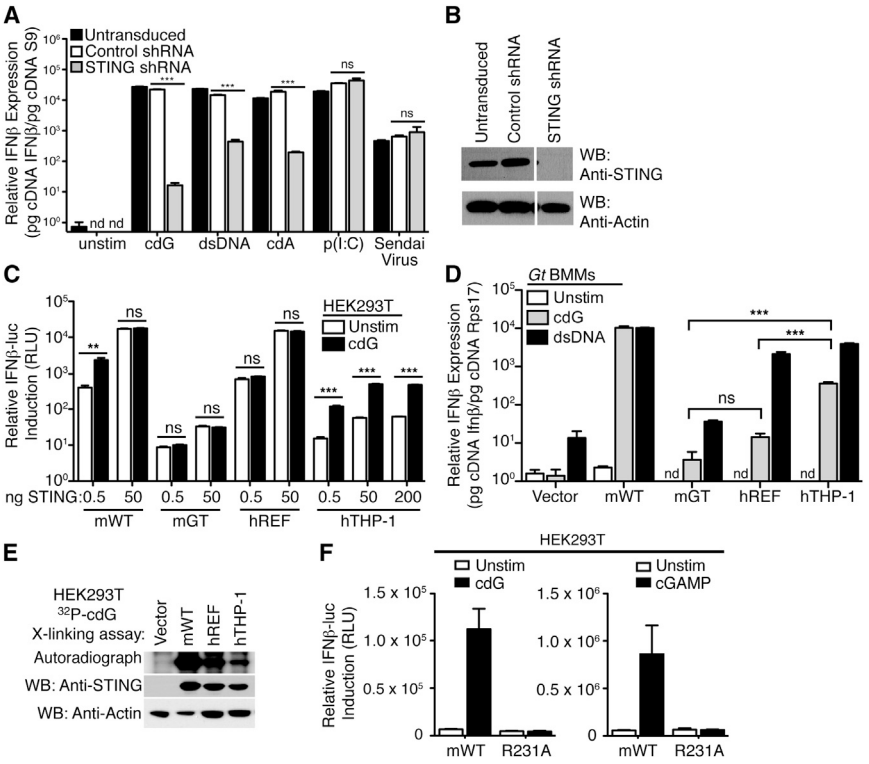

Figure 1. Variable Responsiveness of hSTING Variants to Cyclic Dinucleotides Maps to Arginine 232

(A) THP-1 cells were transduced with vectors encoding an shRNA targeting STING or a control shRNA. Then, cells were stimulated with cyclic di-GMP (cdG), dsDNA, cyclic di-AMP (cdA), polyinosine:cytosine (p(I:C)), or Sendai virus, and the induction of human interferon-β messenger RNA was assessed by qRT- PCR.

(B) Western blotting confirmed that knockdown of STING was effective.

(C) HEK 293T cells were transfected with the indicated amounts of various mouse (m) or human (h) STING expression plasmid and then stimulated 6 hr later by transfection with synthetic cdG (5 μM). GT denotes the null I199N allele of STING from *Goldenticket* (*Gt*) mice. STING activation was assessed with the use of a cotransfected IFNβ-luciferase reporter construct.

(D) *Gt* (STING-null) macrophages were transduced with retroviral vectors encoding the indicated STING alleles and were stimulated 48 hr later by transfection with cdG (5 μM) or dsDNA 70-mer oligonucleotide (0.5 μg/ml). IFNβ induction was measured by qRT-PCR. ND, not detected.

(E) Binding assay of STING to ^{32}P-cdG. STING proteins were expressed in HEK 293T cells, and cell lysates were subjected to UV crosslinking with ^{32}P-cdG and resolved by SDS-PAGE. Binding was quantified by autoradiography. Western blots of cell lysates with an anti-STING polyclonal antibody confirmed similar expression of the various STING proteins.

(F) Responsiveness of mSTING to cGAMP is affected by mutations of R231. The indicated mutants were tested as in (C).

Data are representative of at least three independent experiments and are presented as the mean. Error is represented as SEM. ***$p < 0.0001$, **$p < 0.005$. See also Figure S1.

(Sun et al., 2013; Wu et al., 2013), we found that the human THP-1 cell line responds robustly to CDNs in a manner dependent on STING (Figures 1A and 1B). We cloned an hSTING allele from THP-1 cells and compared its amino acid sequence to the previously widely studied reference allele (NP_938023.1, denoted here as hSTING^{REF}) (Ishikawa and Barber, 2008) (Figure S1). We found that hSTING^{REF} and hSTING^{THP-1} differ at four amino acid positions. Notably, hSTING^{THP-1} encodes an arginine (R) at position 232, corresponding to R231 in mSTING; in contrast, hSTING^{REF} encodes a histidine (H) at position 232. We decided to test the functionality of individual STING alleles by expressing these alleles in human embryonic kidney (HEK) 293T cells that lack endogenous STING. As previously observed (Burdette et al., 2011), overexpression of mSTING in HEK 293T cells induces ligand-independent activation of an IFNβ-luciferase reporter construct, but expression of lower amounts of mSTING renders HEK 293T cells responsive to CDNs (Figure 1C). Similarly, HEK 293T cells overexpressing hSTING^{REF} spontaneously activated an IFNβ-luciferase reporter (Figure 1C). However, unlike mSTING, cells expressing low amounts of hSTING^{REF} were poorly responsive to stimulation with cyclic di-GMP (cdG) (Figure 1C). In contrast, cells expressing hSTING^{THP-1} were responsive to cdG and exhibited ~10-fold induction of the IFN-luciferase reporter, similar to what is seen with mSTING (Figure 1C).

HEK 293T cells expressing STING do not respond to stimulation by dsDNA, presumably because of the lack of expression of cGAS (Sun et al., 2013) or perhaps other dsDNA sensors. Therefore, to test whether the hSTING variants could respond to dsDNA stimulation, we transduced cGAS$^+$ STING-null (*Goldenticket*) (Sauer et al., 2011) macrophages with hSTING expression vectors. Even the hSTING^{REF} variant that responds poorly to CDNs conferred responsiveness to dsDNA (Figure 1D). Therefore, hSTING^{REF} phenocopies mSTING^{R231A} and uncouples responsiveness to CDNs and dsDNA (Burdette et al., 2011).

Consistent with the above results with hSTING variants, an R231H mutant of mSTING responded poorly to CDNs, as did R232A or R232H variants of hSTING^{THP-1} (Figure S2A). Thus, R231 and R232 appear critical for responsiveness to CDNs in mSTING and hSTING, respectively. Introduction of an H232R mutation in hSTING^{REF} was not sufficient to restore responsiveness to CDNs; indeed, we found that a second substitution (G230A) was also required (Figure S3). All the variant STING alleles that we tested bound cdG (Figures 1E and S2B) (Huang et al., 2012; Ouyang et al., 2012; Yin et al., 2012), consistent with the fact that residues 230 and 232 are located in loops that cover, but do not form, the CDN binding pocket.

Importantly, mSTING^{R231A} also failed to respond to chemically synthesized cGAMP (Figure 1F) (Kellenberger et al., 2013). This

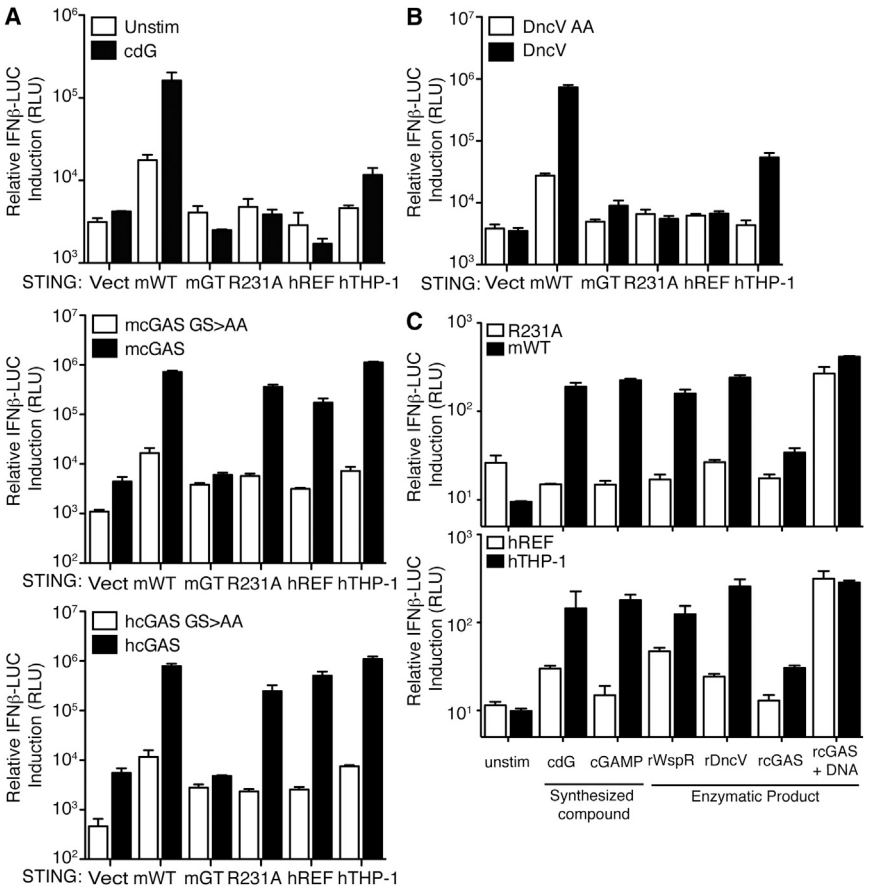

Figure 2. STING Variants Are Responsive to cGAS

(A) HEK 293T cells were transfected with the indicated STING alleles and with human and mouse cGAS (WT and GS > AA mutants) (Sun et al., 2013) as indicated. STING activation was assessed by a cotransfected IFNβ-luciferase reporter construct.

(B) HEK 293T cells were transfected with the indicated STING alleles and with a mammalian expression vector encoding a cGAMP synthase (DncV) from *V. cholerae*. STING activation was assessed as in (A).

(C) In vitro enzymatically generated products of rWspR, rDncV, and rcGAS were transfected into digitonin permeabilized HEK 293T cells expressing the indicated mouse and hSTING proteins. Chemically synthesized cdG and cGAMP were included as controls. STING activation was assessed as in (A) and (B). Data are representative of at least three independent experiments and are presented as mean. Error is represented as SEM. See also Figures S2 and S3.

observation raised the question of whether R231A or R232H variants of STING would respond to the cGAS enzyme that is believed to activate STING via the production of cGAMP. Surprisingly, we found that human or mouse cGAS expression robustly activated hSTING^{REF} and mSTING^{R231A} variants (Figure 2A). We considered several explanations for this puzzling result. One possibility is that the response was due simply to the overexpression of the cGAMP synthase in mammalian cells; however, overexpression of DncV, a bacterial cGAMP synthase from *V. cholerae* (Davies et al., 2012), did not activate hSTING^{REF} or mSTING^{R231A} but did activate wild-type mSTING and hSTING^{THP-1} (Figure 2B). An alternative hypothesis is that cGAS might activate STING by a direct physical interaction and in a manner independent of cGAMP production. However, this explanation also appears to be incorrect. As previously demonstrated (Sun et al., 2013), the overexpression of catalytically dead mutants of human or mouse cGAS (GS > AA; Figure 2A) failed to activate STING variants, arguing that cGAS signaling depends on the production of a second messenger rather than on a direct physical interaction with STING. To confirm this interpretation, we produced the enzymatic product of cGAS by providing ATP, guanosine triphosphate (GTP), and dsDNA to purified recombinant cGAS in vitro. As a negative control, dsDNA (required to stimulate cGAS activity) was omitted from a parallel reaction. Then, the resulting cGAS products were purified and transfected into HEK 293T cells expressing STING variants. In contrast to synthetic cGAMP, the cGAS product was able to activate hSTING^{REF} and mSTING^{R231A} (Figure 2C). This experiment provides evidence against a model in which cGAS activates hSTING^{REF} via a direct physical interaction.

cGAS is structurally homologous to another innate immune sensor called oligoadenylate synthase that produces a noncanonical oligoadenylate polymer containing 2′-5′ phosphodiester bonds (Kranzusch et al., 2013; Sun et al., 2013). Therefore, we hypothesized that cGAS might not produce a canonical CDN as previously proposed (Sun et al., 2013; Wu et al., 2013) but, instead, might produce a CDN containing 2′-5′ phosphodiester bond(s) that could stimulate variant STING alleles. Such a noncanonical CDN would have a mass identical to the canonical 3′-5′ phosphodiester-linked CDN, and, thus, the two products would not have been easy to distinguish by previously published mass spectrometric analyses of the cGAS product (Sun et al., 2013; Wu et al., 2013). We tested our hypothesis by providing radiolabelled α-^{32}P-GTP or α-^{32}P-ATP to recombinant purified cGAS or *V. cholerae* DncV and analyzing the products by thin-layer chromatography (TLC). As reported previously, DncV can produce some cyclic di-AMP (cdA) when provided with only ATP and some cdG when provided with only GTP but prefers to make cGAMP when provided with both ATP and GTP (Davies et al., 2012) (Figure 3A). cGAS requires both ATP and GTP substrates, and the resulting product migrates significantly differently than any of the canonical CDNs produced by DncV, suggesting that cGAS produces a noncanonical CDN (Figure 3A).

We analyzed the cGAS and DncV products by specific nuclease digestion. The cGAS product is partially cleaved by nuclease P1, which selectively digests 3′-5′ phosphodiester

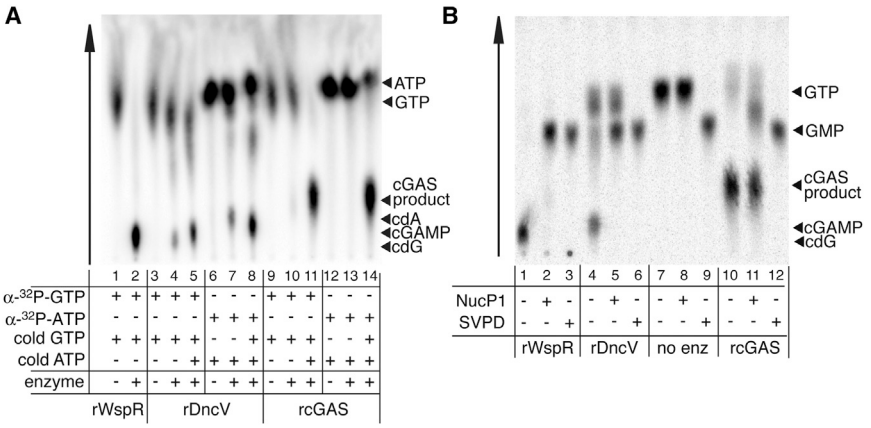

Figure 3. cGAS Produces a Noncanonical Cyclic Dinucleotide

(A) Purified recombinant WspR, DncV, or cGAS were mixed with α-^{32}P-ATP or α-^{32}P-GTP and the indicated unlabeled nucleotides. Reactions were mixed with TLC running buffer, and nucleic acid species were resolved on a polyethylenimine (PEI) cellulose TLC plate.

(B) WspR, DncV, or cGAS products labeled with α-^{32}P-GTP were digested with nuclease P1 or snake venom phosphodiesterase (SVPD) and resolved on a PEI cellulose TLC plate.

linkages (Pino et al., 2008), suggesting that the cGAS product contains at least one 3′-5′ phosphodiester linkage (Figure 3B). Nuclease P1 digestion is incomplete, given that it does not lead to the generation of GMP, in contrast to what is observed upon treatment of the DncV product with nuclease P1 (Figure 3B). As a control, digestion of the cGAS or DncV products with snake venom phosphodiesterase, which cleaves both 2′-5′ and 3′-5′ phosphodiester linkages (Pino et al., 2008), led to complete digestion (Figure 3B). Altogether, these results suggest that the cGAS product might also contain a 2′-5′ phosphodiester linkage.

To identify the nature of the cGAS product and, in particular, ascertain the regiochemistry of the phosphodiester linkages, we analyzed the cGAS product by nuclear magnetic resonance (NMR) spectroscopy (Figures 4A and S4). These results indicate that the product of cGAS is a noncanonical CDN containing a single 2′-5′ phosphodiester linkage and that its chemical structure is assigned as cyclic [G(2′-5′)pA(3′-5′)p] (Figure 4B; see Extended Discussion). This second messenger appears to be a robust activator of STING. A recently published crystallographic and enzymatic study of cGAS independently identified this noncanonical CDN as the product of cGAS (Gao et al., 2013) but did not address its unique signaling properties. Our results demonstrating the unique ability of cyclic [G(2′-5′)pA(3′-5′)p] to stimulate diverse hSTING alleles may explain why cGAS synthesizes this unusual molecule. Although the bacterial cGAMP synthase DncV is also a distant homolog of OAS1 and cGAS (Davies et al., 2012), our IFNβ-luciferase reporter and TLC data strongly suggest that DncV produces a canonical CDN with two 3′-5′ linkages (Figure 4B). It remains to be determined whether any bacterial enzymes produce noncanonical CDNs or whether noncanonical CDNs are unique to mammals.

We identified the R231A mutant of mSTING after a thorough site-directed mutagenesis of STING (Burdette et al., 2011). We were surprised to discover that the corresponding arginine (R232) is a site of natural polymorphism in hSTING. It is tempting to speculate that the R232H variant of hSTING may confer a selective advantage by reducing responses to bacterial CDNs while still retaining responsiveness to endogenous noncanonical CDNs produced by cGAS in response to viral dsDNA. Indeed, although the production of type I IFNs is essential for the control of most viruses, type I IFNs have often been found to be detrimental in the response to bacterial infections (Monroe et al., 2010). It is not yet clear why the R231A and R232H STING variants can be stimulated by the noncanonical CDN and not by the canonical CDNs. An explanation awaits high-resolution structures of these variants bound to cyclic [G(2′-5′)pA(3′-5′)p].

CDNs have been proposed to be useful as vaccine adjuvants or immunotherapeutics (Chen et al., 2010). In addition, a synthetic STING activator, DMXAA, has been tested in human clinical trials as a chemotherapeutic agent. DMXAA was not found to be effective in humans, most likely because it is unable to stimulate hSTING (Conlon et al., 2013). In this context, our results may be significant because they suggest that noncanonical 2′-5′ linked CDNs might have clinical value as potent pan-agonists of diverse STING variants, including those variants that respond poorly to canonical CDNs or DMXAA.

EXPERIMENTAL PROCEDURES

Mice and Cell Lines

THP-1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, and L-glutamine. HEK 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin-streptomycin, and L-glutamine. GP2 retroviral packaging cell lines were maintained in DMEM supplemented with 10% FBS, penicillin-streptomycin, and L-glutamine. Animal protocols were approved by the University of California, Berkeley, Animal Care and Use Committee.

STING Knockdown

Knockdown of hSTING (clone ID NM_198282.1-901s1c1) was achieved with pLKO.1 (The RNAi Consortium). The sequence for the knockdown of hSTING is 5′-GCA GAG CTA TTT CCT TCC ACA, which corresponds to 5′-CCG GGC AGA GCT ATT TCC TTC CAC ACT CGA GTG TGG AAG GAA ATA GCT CTG CTT TTT G forward oligo and 5′-AAT TCA AAA AGC AGA GCT ATT TCC TTC CAC ACT CGA GTG TGG AAG GAA ATA GCT CTG C reverse oligo. Oligos were annealed and cloned into AgeI and EcoRI digested pLKO.1 (Addgene) and retrovirally transduced into THP-1 cells in parallel with scramble small hairpin RNA (shRNA) control constructs. Stable cell lines were selected with puromycin. THP-1 cells were differentiated with 1 μg/ml PMA for 24 hr. Cells were allowed to rest for 24 hr and then restimulated for 6 hr with the indicated ligands. IFNβ induction was measured by quantitative RT-PCR (qRT-PCR) as described below.

Cell Stimulation and Reagents

Bone marrow macrophages and HEK 293T cells were stimulated with Lipofectamine 2000 (Invitrogen). Unless otherwise specified, cdG, cdA, poly(I:C), and vaccinia virus 70-mer DNA were prepared as described

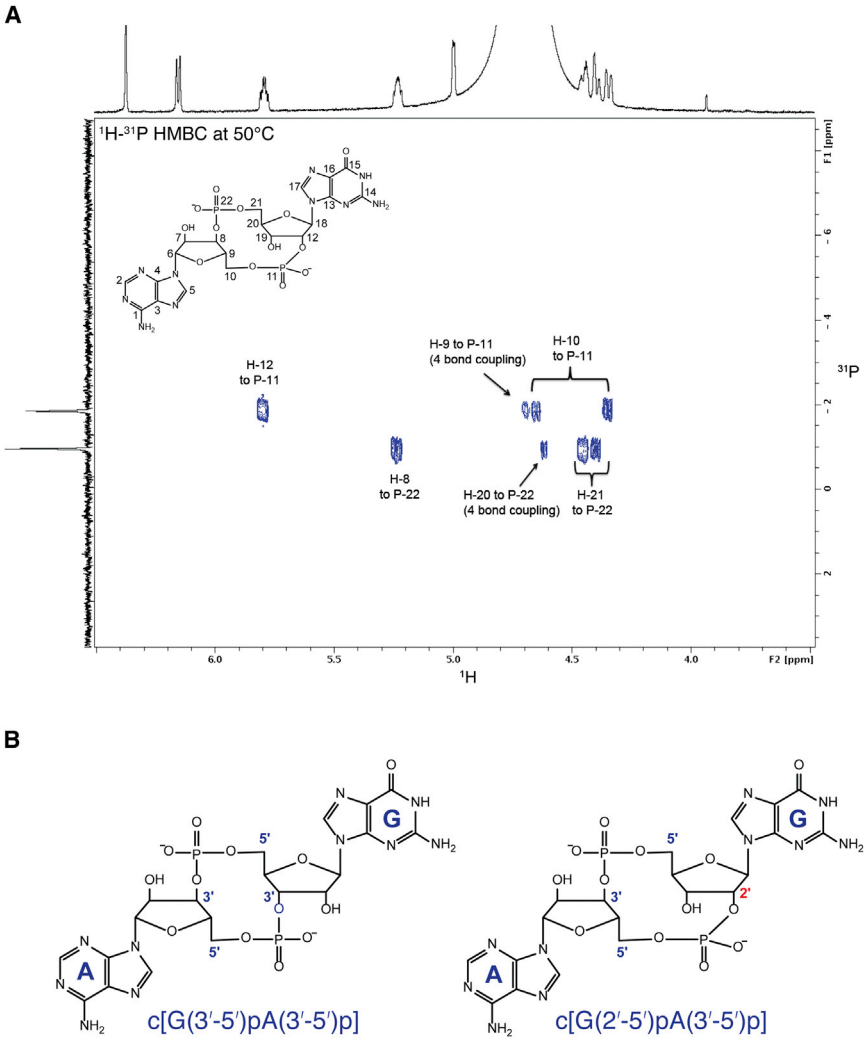

Figure 4. cGAS Produces a Cyclic Dinucleotide Containing a 2′-5′ Phosphodiester Linkage

(A) ^{1}H-^{31}P HMBC of HPLC-purified cGAS product acquired at 600 MHz and 50°C. Critical through-bond correlations for the phosphodiester bonds are indicated. An NMR-elucidated structure of the cGAS product is also shown.

(B) Chemical structures of canonical cGAMP, cyclic [G(3′-5′)pA(3′-5′)p] (left), and noncanonical cGAMP, cyclic [G(2′-5′)pA(3′-5′)p] (right) are shown.

See also Figure S4.

previously (Burdette et al., 2011) and used at similar concentrations. Sendai virus was purchased from Charles River Laboratories. cGAMP was synthesized as previously described (Kellenberger et al., 2013).

Cloning, Mutagenesis, and Plasmids

The THP-1 STING allele was amplified from complementary DNA (cDNA) with 5′ hSTING HindIII (5′-ATC GAA GCT TCC ACC ATG CCC CAC TCC AGC CTG) and 3′ hSTING NotI (5′-ATC GGC GGC CGC TCA GGC ATA GTC AGG CAC GTC ATA AGG ATA AGA GAA ATC CGT GCG GAG AG). The resulting PCR product was cloned into pCDNA3 with HindIII-NotI digestion. THP-1 STING was amplified and cloned into MSCV2.2 with the 3′ primer listed above and 5′ hSTING XhoI (5′-ATC GCT CGA GCC ACC ATG CCC CAC TCC AGC CTG) and XhoI-NotI digestion. IFNβ-luciferase, TK-*Renilla*, and mSTING plasmids were used as previously described (Burdette et al., 2011). Mutations in hSTING were introduced with a QuikChange Site-Directed Mutagenesis Kit (Stratagene). cDNA clones corresponding to mouse and human cGAS (MGC Fully Sequenced Human MB21D1 cDNA: accession, BC108714.1 and clone ID, 6015929; EST Fully Sequenced Mouse E330016A19Rik cDNA: accession, BC145653.1 and clone ID, 40130956) were obtained from Open Biosystems and correspond to those described previously (Sun et al., 2013; Wu et al., 2013). Mouse cGAS was amplified from cDNA clones with an N-terminal flag tag with forward oligo 5′-mcGAS-KpnI (5′-ATC GGG TAC CCC ACC ATG GAT TAC AAG GAT GAC GAT GAC AAG GAA GAT CCG CGT AGA AGG) and reverse oligo 3′-mcGAS-NotI (5′-ATC GGC GGC CGC TCA AAG CTT GTC AAA AAT TGG). Likewise, hcGAS was amplified with forward oligo 5′-hcGAS-flag-KpnI (5′-ATC GGG TAC CCC ACC ATG GAT TAC AAG GAT GAC GAT GAC AAG CAG CCT TGG CAC GGA AAG G) and reverse 3′-hcGAS-NotI (5′-ATC GGC GGC CGC TCA AAA TTC ATC AAA AAC TGG AAA C). Both PCR products were cloned into pcDNA3 at KpnI and NotI restriction enzyme sites. DncV was amplified with DncV forward BamHI (5′-GCA TGG ATC CGC CAC CAT GAC TTG GAA CTT TCA CCA G) and DncV reverse NotI (5′-GCA TGC GGC CGC TCA GCC ACT TAC CAT TGT GCT GC) and cloned into pCDNA3 with BamHI and NotI. For cloning into MSCV2.2, DncV was amplified with DncV forward XhoI (5′-GCA TCT CGA GCC ACC ATG ACT TGG AAC TTT CAC CAG) and DncV reverse NotI. Resulting DNA was cloned into MSCV 2.2 digested with XhoI-NotI. Constructs for bacterial mcGAS overexpression were constructed as follows. N-terminal His6-SUMO tag amplified by PCR with His6 SUMO NcoI (5′-TAA TAA GGA GAT ATA CCA TGG GCA GCA GCC) and His6 SUMO SalI (5′-GAA TTC GTC GAC ACC AAT CTG TTC TCT GTG AGC) off of a pCDF-Duet2 template (a gift from the M. Rape lab) and cloned into pET28a with NcoI and SalI to make pET28a-H6SUMO. Full-length mcGAS was PCR amplified from the mouse cDNA clone described above with mcGAS forward SalI (5′-GAT GTC GAC ATG GAA GAT CCG CGT AGA AGG ACG) and mcGAS reverse XhoI (5′-ATC CTC GAG TCA AAG CTT GTC AAA AAT TGG AAA CC) and cloned into pET28a-H6SUMO with SalI and XhoI to make pET28a-H6SUMO-mcGAS that expresses full-length mcGAS fused to an N-terminal His6 SUMO tag.

cGAS Product Purification and Structural Characterization

The cGAS product (prepared in vitro as described below) was purified with reverse-phase high-performance liquid chromatography (HPLC) on an Agilent 1260 Infinity HPLC equipped with an Agilent Polaris C18-A column (5 μm, 250 mm × 10 mm, 180 Å). Purification conditions included a 100% to 0% gradient of solvent A over 20 min at 50°C and a flow rate of 5 ml/min, solvent A being 100 mM ammonium acetate in water at pH 7 and solvent B being acetonitrile. Purified elution fractions were evaporated multiple times in order to remove excess ammonia.

Resonance assignments were made with COSY, ^{1}H-^{13}C HSQC, NOESY, ^{1}H-^{13}C HMBC, and ^{1}H-^{31}P HMBC.

The characterization of cGAS product is as follows. ^{1}H NMR (900 MHz, D_2O, 50°C, δ): 8.44 (s, 1), 8.42 (s, 1), 8.03 (s, 1), 6.31 (s, 1), 6.09 (d, 1, J = 8 Hz), 5.75 (m, 1), 5.18 (m, 1), 4.93 (s, 1), 4.74, 4.62, 4.59 (d, 1, J = 12 Hz), 4.55 (s, 1), 4.38 (m, 1), 4.33 (d, 1, J = 12 Hz), 4.28 (d, 1, J = 12 Hz); ^{31}P {^{1}H

decoupled} NMR (600 MHz, D_2O, 50°C, δ): (all resonances are singlets) −0.96, −1.86; HRMS (*m/z*): [M-H$^+$] calculated for $C_{20}H_{23}N_{10}O_{13}P_2$, 673.0927; found, 673.0909. [M + Na$^+$ − 2H$^+$] calculated for $C_{20}H_{22}N_{10}O_{13}P_2Na$, 695.0746; found, 695.0728.

Luciferase Assay

HEK 293T cells were plated in TC-treated 96-well plates at 0.5 × 10^6 cells ml^{-1}. The next day, the cells were transfected with indicated constructs, along with IFN-β firefly luciferase and TK-*Renilla* luciferase reporter constructs. After stimulation for 6 hr with the indicated ligands, the cells were lysed in passive lysis buffer (Promega) for 5 min at 25°C. The cell lysates were incubated with firefly luciferase substrate (Biosynth) and the *Renilla* luciferase substrate coelenterazine (Biotium), and luminescence was measured on a SpectraMax L Luminescence Microplate Reader (Molecular Devices). The relative IFNB expression was calculated as firefly luminescence relative to *Renilla* luminescence. Statistical differences were calculated with an unpaired two-tailed Student's t test with the use of Prism 5.0b software (GraphPad).

In Vitro Cyclic Dinucleotide Synthesis

In vitro DncV reactions were carried out in 20 mM Tris-Cl (pH 8), 20 mM $Mg(OAc)_2$, 10% glycerol, and 1 mM dithiothreitol, 0.1 mg/ml BSA. Reactions contained 250 μM GTP and 250 μM ATP or 125 μM GTP and 125 μM ATP, as indicated in the figures. In addition, 33 nM α-^{32}P-GTP (3,000 Ci/mmol, PerkinElmer) or 33 nM α-^{32}P-ATP (3,000 Ci/mmol, PerkinElmer) was included in reaction where indicated. Reactions were started by the addition of 1 μM purified DncV protein. In vitro cGAS reactions were carried out in 40 mM Tris-Cl (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$. Cold nucleotide and α-^{32}P-GTP was at the same concentrations as in DncV reactions. Reactions were started by the addition of 200 nM purified cGAS. Where indicated, herring testes DNA (Sigma-Aldrich) was added to reactions at a final concentration of 0.1 mg/ml. WspR reactions were performed as described previously (Burdette et al., 2011). Reactions were incubated for 1 hr at 37°C, boiled for 5 min at 95°C, and spun for 10 min at 13,000 rpm. Reactions were removed and mixed 1:5 with TLC running buffer (1:1.5 [v/v] saturated NH_4SO_4 and 1.5 M KH_2PO_4 [pH 3.6]) and spotted on polyethylenimine cellulose TLC plate (Sigma-Aldrich). Following solvent migration, the TLC plate was exposed to a phosphorimager screen and imaged with a Typhoon scanner. For in vitro product transfection into HEK 293T cells, reactions were scaled up, radiolabeled nucleotide was omitted, and the concentration of ATP and GTP was increased to 2 mM.

ACCESSION NUMBERS

The GenBank accession number for the STING^{THP-1} sequence reported in this paper is KF029721.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, Extended Discussion, and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.05.009.

LICENSING INFORMATION

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REFERENCES

Barbalat, R., Ewald, S.E., Mouchess, M.L., and Barton, G.M. (2011). Nucleic acid recognition by the innate immune system. Annu. Rev. Immunol. *29*, 185–214.

Barker, J.R., Koestler, B.J., Carpenter, V.K., Burdette, D.L., Waters, C.M., Vance, R.E., and Valdivia, R.H. (2013). STING-Dependent Recognition of Cyclic di-AMP Mediates Type I Interferon Responses during Chlamydia trachomatis Infection. mBio *4*, 00018–00013.

Burdette, D.L., Monroe, K.M., Sotelo-Troha, K., Iwig, J.S., Eckert, B., Hyodo, M., Hayakawa, Y., and Vance, R.E. (2011). STING is a direct innate immune sensor of cyclic di-GMP. Nature *478*, 515–518.

Chen, W., Kuolee, R., and Yan, H. (2010). The potential of 3′,5′-cyclic diguanylic acid (c-di-GMP) as an effective vaccine adjuvant. Vaccine *28*, 3080–3085.

Conlon, J., Burdette, D.L., Sharma, S., Bhat, N., Thompson, M., Jiang, Z., Rathinam, V.A., Monks, B., Jin, T., Xiao, T.S., et al. (2013). Mouse, but not Human STING, Binds and Signals in Response to the Vascular Disrupting Agent 5,6-Dimethylxanthenone-4-Acetic Acid. J. Immunol. *190*, 5216–5225.

Davies, B.W., Bogard, R.W., Young, T.S., and Mekalanos, J.J. (2012). Coordinated regulation of accessory genetic elements produces cyclic di-nucleotides for V. cholerae virulence. Cell *149*, 358–370.

Gao, P., Ascano, M., Wu, Y., Barchet, W., Gaffney, B.L., Zillinger, T., Serganov, A.A., Liu, Y., Jones, R.A., Hartmann, G., et al. (2013). Cyclic [G(2′,5′)pA(3′,5′)p] Is the Metazoan Second Messenger Produced by DNA-Activated Cyclic GMP-AMP Synthase. Cell.

Huang, Y.H., Liu, X.Y., Du, X.X., Jiang, Z.F., and Su, X.D. (2012). The structural basis for the sensing and binding of cyclic di-GMP by STING. Nat. Struct. Mol. Biol. *19*, 728–730.

Ishii, K.J., Coban, C., Kato, H., Takahashi, K., Torii, Y., Takeshita, F., Ludwig, H., Sutter, G., Suzuki, K., Hemmi, H., et al. (2006). A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nat. Immunol. *7*, 40–48.

Ishikawa, H., and Barber, G.N. (2008). STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature *455*, 674–678.

Ishikawa, H., Ma, Z., and Barber, G.N. (2009). STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature *461*, 788–792.

Jin, L., Hill, K.K., Filak, H., Mogan, J., Knowles, H., Zhang, B., Perraud, A.L., Cambier, J.C., and Lenz, L.L. (2011a). MPYS is required for IFN response factor 3 activation and type I IFN production in the response of cultured phagocytes to bacterial second messengers cyclic-di-AMP and cyclic-di-GMP. J. Immunol. *187*, 2595–2601.

Jin, L., Xu, L.G., Yang, I.V., Davidson, E.J., Schwartz, D.A., Wurfel, M.M., and Cambier, J.C. (2011b). Identification and characterization of a loss-of-function human MPYS variant. Genes Immun. *12*, 263–269.

Kranzusch, P.J., Lee, A.S.Y., Berger, J.M., and Doudna, J.A. (2013). Structure of human cGAS reveals a conserved family of second messenger enzymes in innate immunity. Cell Rep *3*, this issue, 1362–1368.

Kellenberger, C.A., Wilson, S.C., Sales-Lee, J., and Hammond, M.C. (2013). RNA-Based Fluorescent Biosensors for Live Cell Imaging of Second Messengers Cyclic di-GMP and Cyclic AMP-GMP. J. Am. Chem. Soc.

McWhirter, S.M., Barbalat, R., Monroe, K.M., Fontana, M.F., Hyodo, M., Joncker, N.T., Ishii, K.J., Akira, S., Colonna, M., Chen, Z.J., et al. (2009). A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. J. Exp. Med. *206*, 1899–1911.

Monroe, K.M., McWhirter, S.M., and Vance, R.E. (2010). Induction of type I interferons by bacteria. Cell. Microbiol. *12*, 881–890.

Ouyang, S., Song, X., Wang, Y., Ru, H., Shaw, N., Jiang, Y., Niu, F., Zhu, Y., Qiu, W., Parvatiyar, K., et al. (2012). Structural analysis of the STING adaptor protein reveals a hydrophobic dimer interface and mode of cyclic di-GMP binding. Immunity *36*, 1073–1086.

Palm, N.W., and Medzhitov, R. (2009). Pattern recognition receptors and control of adaptive immunity. Immunol. Rev. *227*, 221–233.

Pino, S., Ciciriello, F., Costanzo, G., and Di Mauro, E. (2008). Nonenzymatic RNA ligation in water. J. Biol. Chem. *283*, 36494–36503.

Sauer, J.D., Sotelo-Troha, K., von Moltke, J., Monroe, K.M., Rae, C.S., Brubaker, S.W., Hyodo, M., Hayakawa, Y., Woodward, J.J., Portnoy, D.A., and Vance, R.E. (2011). The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to Listeria monocytogenes and cyclic dinucleotides. Infect. Immun. *79*, 688–694.

Stetson, D.B., and Medzhitov, R. (2006). Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. Immunity *24*, 93–103.

Sun, W., Li, Y., Chen, L., Chen, H., You, F., Zhou, X., Zhou, Y., Zhai, Z., Chen, D., and Jiang, Z. (2009). ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. Proc. Natl. Acad. Sci. USA *106*, 8653–8658.

Sun, L., Wu, J., Du, F., Chen, X., and Chen, Z.J. (2013). Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science *339*, 786–791.

Takeuchi, O., and Akira, S. (2010). Pattern recognition receptors and inflammation. Cell *140*, 805–820.

Woodward, J.J., Iavarone, A.T., and Portnoy, D.A. (2010). c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. Science *328*, 1703–1705.

Wu, J., Sun, L., Chen, X., Du, F., Shi, H., Chen, C., and Chen, Z.J. (2013). Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. Science *339*, 826–830.

Yin, Q., Tian, Y., Kabaleeswaran, V., Jiang, X., Tu, D., Eck, M.J., Chen, Z.J., and Wu, H. (2012). Cyclic di-GMP sensing via the innate immune signaling protein STING. Mol. Cell *46*, 735–745.

Zhong, B., Yang, Y., Li, S., Wang, Y.Y., Li, Y., Diao, F., Lei, C., He, X., Zhang, L., Tien, P., and Shu, H.B. (2008). The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. Immunity *29*, 538–550.