

## CHARACTERIZATION OF STIMULATOR OF INTERFERON GENES (STING) EXPRESSION IN HUMAN EPIDERMAL KERATINOCYTES

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**Abstract** The innate immune system is the first line of defense against microbial pathogens, and the production and secretion of interferons (IFNs) plays an important role of the innate response to viral infections. Recently stimulator of interferon genes (STING) has been identified as an intracellular adaptor, which activates both NF- $\kappa$ B and interferon regulatory factor 3 (IRF3) transcription pathways to induce IFNs. We demonstrated that the STING expression is regulated by stimulation of various cytokines in human epidermal keratinocytes (HaCaT). Especially, IFN- $\gamma$  induced the STING mRNA expression in a dose-dependent manner. On the other hand, polyinosinic-polycytidylic acid (poly I:C), which mimics viral infection, has no effect on the STING expression. STING promoter analysis showed that a gamma-activated sequence (GAS) located at -58 to -66 bp is essential for IFN- $\gamma$ -regulated promoter activity. Moreover, electrophoretic mobility shift assay revealed that STAT1 binds to the GAS element on the STING promoter region, indicating that IFN- $\gamma$ /JAK/STAT1 signaling is critical for the expression of STING in human epidermal keratinocytes.

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**Key words:** STING; IFN- $\gamma$ ; STAT1; HaCaT

### Introduction

Host exposure to microbial pathogens such as viruses, bacteria, and fungi trigger the activation of innate immune responses that stimulate early host defense mechanisms such as the production of IFNs. The pattern recognition receptors (PRRs) responsible for sensing viral RNA have recently been isolated and have been found to involve DExD/H box RNA helicases called retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5). These helicases are responsible for the production of type I IFN in all cell types, in response to RNA virus infection. Helicase interaction with viral RNA induces the recruitment of a molecule referred to as IPS-1 (also known as VISA, Cardif or MAVS) and TRAF3 and leads to the activation of mitogen activated protein (MAP)

kinases, the IKK-related kinases, TBK1 (TANK binding kinase 1) and IKK-I (I $\kappa$ B kinase I). These kinases activate the transcription factors NF- $\kappa$ B, activator protein-1 (AP1) and interferon regulatory factor-3 (IRF3), which translocate into the nucleus and bind to and activate the type I IFN promoter<sup>1)</sup>. IFN is secreted, binds to the type I IFN receptor (IFNAR) in an autocrine or paracrine fashion and activates the JAK / STAT pathway to initiate the production of numerous IFN-inducible genes which exert potent anti-viral activity through mechanisms that in many cases remain to be fully clarified<sup>2)</sup>. Recently, a molecule referred to as STING (stimulator of IFN genes), also known as MITA/MPYS/ERIS, was demonstrated as being vital for recognizing cytoplasmic DNA and for activating the production of innate immune genes in response to a variety of DNA pathogens and even certain

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RNA viruses. STING is essential for cytosolic DNA-mediated type I IFNs induction. STING contained multi-putative transmembrane regions in the amino terminal region, and was found to predominantly localize in the endoplasmic reticulum (ER) and the mitochondria. Overexpression of STING induces the activation of both NF- $\kappa$ B and IRF3 to stimulate type I IFN production and stimulating host defenses against pathogens<sup>3</sup>.

We demonstrated that the STING expression is regulated by stimulation of various cytokines in human epidermal keratinocytes (HaCaT). Especially, IFN- $\gamma$  induced the STING mRNA expression in a dose-dependent manner. On the other hand, polyinosinic-polycytidylic acid (poly I:C), which mimics viral infection, has no effect on the STING expression. IFN- $\gamma$  is a proinflammatory cytokine that is secreted by activated T-lymphocytes and natural killer cells and regulates cellular antiviral, antitumor, and immunological responses. IFN- $\gamma$  has been reported to regulate cyclooxygenase-2 (COX-2) expression and induce prostaglandin formation, which may play a major role in the induction of inflammatory processes. IFN- $\gamma$  also induces intercellular adhesion molecule-1 (ICAM-1), which plays an important role in the adherence and migration of leukocytes at sites of inflammation in several cell type. The binding of IFN- $\gamma$  to its surface receptor activates the receptor-associated tyrosine kinases, JAK1 and JAK2<sup>4</sup>. JAKs tyrosine phosphorylate and activate the latent cytosolic transcription factor STAT1, which then dimerizes, translocates to the nucleus, and binds to the  $\gamma$ -activated sequence (GAS) elements of IFN- $\gamma$  response genes, resulting in gene activation<sup>5</sup>. IFN- $\gamma$  has many different biological effects that contribute to the production of proinflammatory cytokines and chemokines, however, the underlying mechanism of IFN- $\gamma$  involvement in STING expression is unknown. In this study

we investigate the effect of IFN- $\gamma$  on STING transcription and expression of STING mRNA in human epidermal keratinocytes.

## Materials and methods

### Cell culture

HaCaT cells, a spontaneously immortalized, nontumorigenic human skin keratinocyte cell line, were maintained in **Dulbecco's modified eagle medium** (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mg/ml sodium hydrogen carbonate, 100 mg/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml amphotericin B. For experiments with cytokines, 60% confluent HaCaT cells were washed twice with sterile phosphate-buffered saline (PBS) and maintained in DMEM with 0.1% FBS prior to addition of recombinant human IFN- $\gamma$  (10 ng/ml), IFN- $\beta$  (10,000 units/ml), TNF- $\alpha$  (10 ng/ml), TGF- $\beta$  (10 ng/ml). Cultures were maintained at 37.8°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

### Real-time PCR

Total RNA was extracted from cultured cells using RNeasy total RNA isolation kit following the manufacturer's protocol (Qiagen, Hilden, Germany). Extracted RNA was subjected to reverse transcription using RNA PCR Kit (AMV) Ver. 3.0 (Takara, Kyoto, Japan), according to the instructions provided by the manufacturer. Primers for STING and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: STING-F (5'-GAGAGCCACCAGAGCACAC-3'), STING-R (5'-CGCACAGTCCTCCAGTAGC-3'), GAPDH-F (5'-CCACCCATGGCAAATTCATGGCA-3'), and GAPDH-R (5'-TCTAGACGGCAGGTCAGGTCCACC-3'). Real-time PCR was performed on a Opti-con2 real-time system (Bio-Rad, Hercules, CA, USA) with the iQ SYBR Green supermix

(Bio-Rad). The PCR was examined with 1.0  $\mu$ l of cDNA in a 20  $\mu$ l reaction mixture of 10  $\mu$ l iQ SYBR Green supermix, 0.2  $\mu$ l primers and 8.6  $\mu$ l PCR grade water. The reaction was performed using primers with a denaturation step of 95°C for 3 min followed by 40 cycles at 95°C for 15 s, 56°C for 30 s.

### Reporter plasmid construction.

Plasmids used for functional analysis of the STING promoter activity were generated by using pGL3 basic vector (Promega, WI, U.S.A) that contains a promoterless luciferase reporter gene. we generated the STING promoter construct (p-854/88; 0.9-kb fragment) by the PCR amplification method. The forward primers contained an internal site for *Xho*I restriction enzyme and their sequences are 5'-TCAGGTACCGAATGAAATCAAGGCACAGAGCAAG-3'. The sequence of the reverse primer contained site for *Kpn*I enzyme was 5'-ACGCTCGAGAGCAGGACTCCACACACTCAGCCAA-3'. PCR products were then digested with *Xho*I and *Kpn*I enzymes and cloned into luciferase reporter gene vector, pGL3 basic (Promega). The fidelity of the constructs was then confirmed by sequencing, and plasmids were prepared for transfection by use of jetPEI<sup>TM</sup> transfection reagent (Polyplus-transfection, Illkirch, France).

### Site mutagenesis

We prepared the mutations in the critical potential binding site [IFN- $\gamma$ -activated sequence (GAS)] for transcription factor STAT1 (-58 to -66 bp), the sequence is 5'-TTACCGGAA-3', in the STING promoter construct. The mutant construct (Mut) containing 4 bp nucleotide substitutions, the sequence is 5'-TGGCCTAAA-3', on GAS element.

### Transient transfection and luciferase assays

HaCaT cells were transfected by using jetPEI<sup>TM</sup> transfection reagent (Polyplus-transfection) according to the manufacturer's instructions. Once 60% confluent, HaCaT cells were transfected with one of the STING promoter-luciferase constructs (1,300 ng/ml) and 10  $\mu$ g/ml of pGL 4.74 (the *renilla* luciferase activities of the internal control vector, Promega). At 48 h posttransfection, the cells were starved in DMEM/0.1% FBS for 12 h prior to stimulation with IFN- $\gamma$  for 12h. Then, the cells were lysed in reporter lysis buffer (Promega) and the activities of both firefly luciferase and the *renilla* luciferase were measured by luminometer according to the manufacturer's instructions with kits from Promega, respectively. The promoter activity was expressed as a ratio of luciferase to the *renilla* luciferase activity (relative luciferase activity) in each sample.

### Electrophoretic mobility shift assay (EMSA)

For EMSA, 25-mer oligonucleotide probes encoding the  $\gamma$ -activated sequence (GAS) were synthesized and designated wtGAS; the sequences were 5'-AGCTCTTGTTTACCGGAAATACTGT-3' (sense strand, -74 to -50 bp) and 5'-GTTATTTCCGGTAACAAGAGCTATT-3' (antisense strand). Underscored is the GAS located from -66 to -58 bp in the STING promoter. The sequences were 3' end labeled separately with biotin. The biotin-labeled double-stranded probe was prepared using an equal molar ratio of purified non-labeled complementary oligos. Oligos were annealed by PCR: 1 cycle of 94°C for 10 min. A mutated probe (mtGAS), which had 4 base changes (underscored), was also prepared. The sequences were 5'

-AGCTCTTGTGGCCTAAAATAACTGT-3' (sense strand) and 5'-ACAGTTATT TTAGGCCACAAGAGCT-3' (antisense strand). Therefore, we purchased specific consensus binding motif for STAT1, Stat1 p84/p91 Consensus Oligonucleotide (Santa Cruz, CA, U.S.A) as control in which one or more nucleotides mapping within the consensus binding site has been substituted. Nuclear extracts were prepared from HaCaT cells treated with IFN- $\gamma$  (10 ng/ml) for 6h using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, IL, U.S.A). EMSA was performed using the LightShift™ Chemiluminescent EMSA Kit (Pierce) following the manufacturer's instructions. Briefly, 3.05  $\mu$ g of nuclear extract protein was incubated with 20 fmol of biotin-labeled probe in 1 $\times$  EMSA Binding Buffer (10 mM Tris, 50 mM KCl, and 1 mM DDT, pH 7.5) containing 50 ng/ $\mu$ l poly (dI·dC). For the competition assay, a 50- and 100-fold molar excess of unlabeled wtGAS and mtGAS probes was added to the binding reactions. For the antibody supershift EMSA, 3.05  $\mu$ g of nuclear extracts was preincubated with 4  $\mu$ g of anti-p-Stat1 (Tyr 701) monoclonal antibody (Santa Cruz) for 3h, at 4°C, prior to the addition of the labeled probe. After incubation for 20 min at room temperature, protein-DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel and transferred onto nylon membrane (Hybond N+; GE Healthcare, Pis-cataway, NJ, U.S.A). The transferred DNA was cross-linked by UV onto the membrane. Detection of biotin-labeled probes was accomplished by using the LightShift Chemiluminescent EMSA Kit (Pierce).

## Results

### Modulation of STING expression by cytokines in human keratinocytes

In this study, we examined the expression of

STING in HaCaT cells treated with IFN- $\gamma$  (10 ng/ml), IFN- $\beta$  (10,000 units/ml), TNF- $\alpha$  (10 ng/ml), TGF- $\beta$  (10 ng/ml) or none for 12 h. As shown in Fig. 1, stimulation by IFN- $\gamma$  significantly increased STING mRNA expression to 4-folds that of unstimulated control HaCaT cells. In contrast, neither IFN- $\beta$ , TNF- $\alpha$ , TGF- $\beta$  nor poly I:C affected STING expression in HaCaT cells (Fig. 1a).

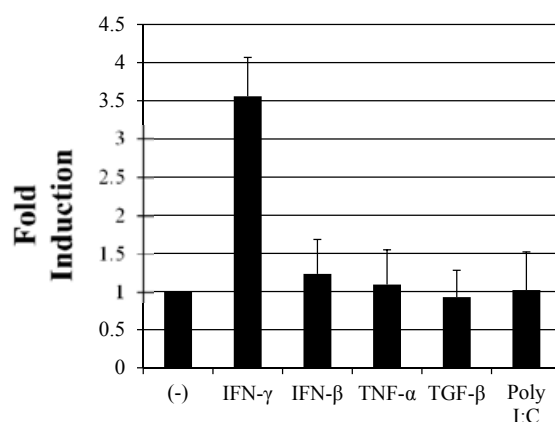
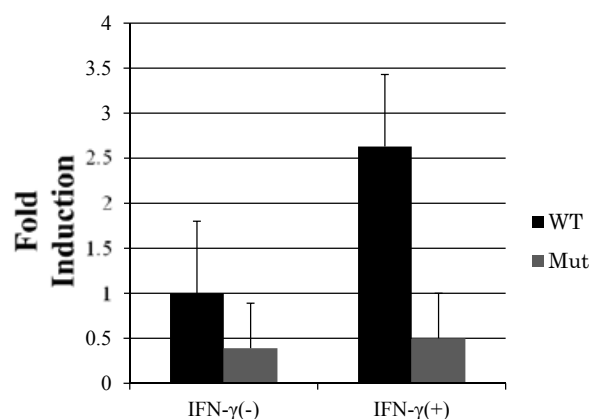
### Activation of Jak/STAT pathway by IFN- $\gamma$ and STAT1 binding to GAS element in STING promoter

One potential GAS was found within the STING promoter. IFN- $\gamma$  is known to activate JAK1, JAK2, and, subsequently, the STAT1 transcriptional factor. We postulated that IFN- $\gamma$ -activated STAT1 acts as an activator of the transcription of STING binding with the GAS motif in the STING gene.

To explore this possibility, we studied the activation of the JAK/STAT signaling pathway. We developed 0.9 kb human STING promoter construct (WT) linked to luciferase reporter gene and mutant construct (Mut) containing 4 bp nucleotide substitutions on GAS element. We transiently transfected these plasmids into HaCaT cells for 6h. Stimulation by IFN- $\gamma$  significantly increased STING promoter activity to 2.5-folds that of unstimulated control in STING promoter construct (WT), while no change was shown in mutant construct (Mut). This finding documents an interaction between GAS element and IFN- $\gamma$ -regulated STING promoter activity (Fig. 1b).

### The GAS element binds to a STAT1-containing complex in HaCaT cells

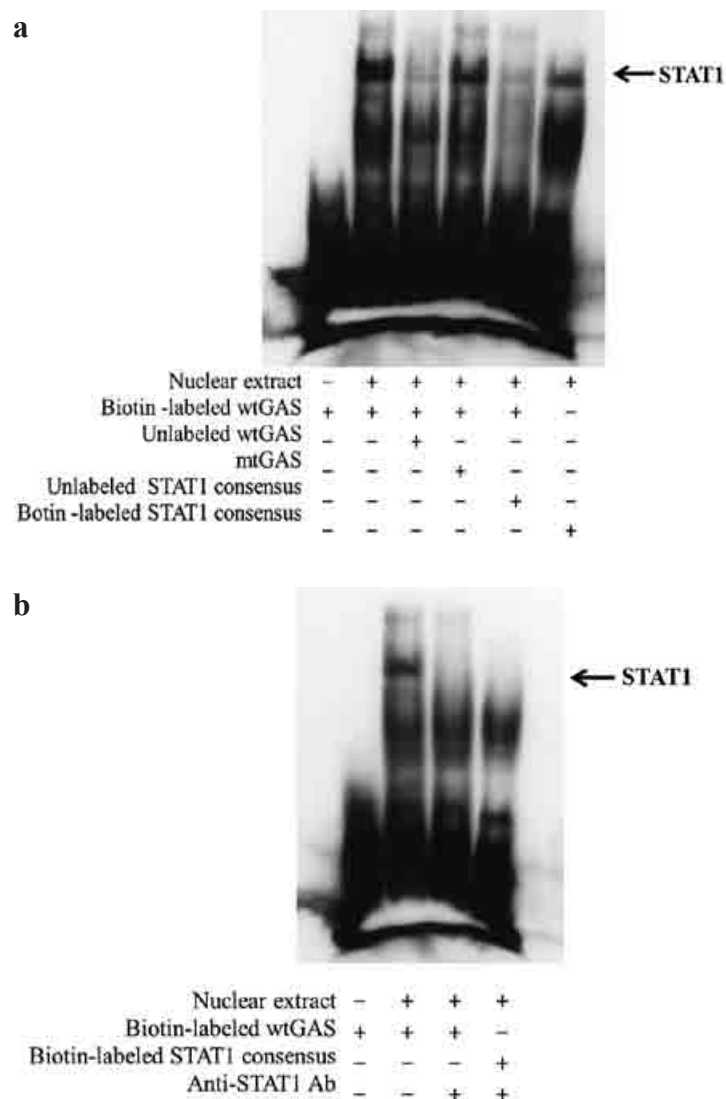
To correlate the studies on promoter activity and to analyze the nature of the complex that binds to the GAS element constitutively, we investigated by electrophoretic mobility shift assay (EMSA) the effects of mutations in the GAS region of STING promoter on tran-

**a****b**

**Fig. 1** Effect of IFN- $\gamma$  on the STING expression in HaCaT cells. HaCaT cells were stimulated with IFN- $\gamma$  (10 ng/ml), IFN- $\beta$  (10,000 units/ml), TNF- $\alpha$  (10 ng/ml), TGF- $\beta$  (10 ng/ml) or none for 12 h. Stimulation by IFN- $\gamma$  significantly increased STING mRNA expression to 4-folds that of unstimulated control. In contrast, neither IFN- $\beta$ , TNF- $\alpha$ , TGF- $\beta$  nor poly I:C affected STING expression in HaCaT cells (a). We developed 0.9 kb human STING promoter construct (WT) linked to luciferase reporter gene and mutant construct (Mut) containing 4 bp nucleotide substitutions on GAS element. We transiently transfected these plasmids into HaCaT cells for 6h. Stimulation by IFN- $\gamma$  significantly increased STING promoter activity to 2.5-folds that of unstimulated control. The analysis showed that GAS element is essential for IFN- $\gamma$ -regulated STING promoter activity (b).

scription factor binding in IFN- $\gamma$ -treated HaCaT cells. These data were compared with constitutive and IFN- $\gamma$ -induced binding to the STING GAS. EMSAs were performed with the following probes: the GAS site and part of the adjoining STING promoter (wtGAS), mutant GAS (mtGAS) probe with 4 bp substitutions on the GAS site and Stat1 p84/p91 Consensus Oligonucleotide (Santa Cruz) as control. The probes were labeled with biotin. Nuclear ex-

tracts from IFN- $\gamma$ -treated HaCaT cells / GAS binding complex can also be detected as well as in case of consensus oligonucleotide. Therefore unlabeled GAS probe, but not mutant GAS probe, inhibited formation of the nuclear extract / labeled GAS probe complex. Thus, EMSA showed the specific binding of the nuclear extracts to GAS probe of the STING promoter in IFN- $\gamma$ -treated HaCaT cells (Fig. 2a). Gel shift supershift analysis showed that anti-STAT1



**Fig. 2** Binding of STAT1 to GAS element of human STING promoter. Nuclear extracts were prepared from HaCaT cells stimulated by IFN- $\gamma$  (10 mg/ml, 6 h). Synthetic GAS-DNA sequence (wtGAS), purchased consensus binding motif for STAT1, and mutant GAS (mtGAS) probe with 4 bp substitutions were used. The probes were labeled with biotin. Unlabeled GAS probe, but not mutant GAS probe, inhibited formation of the nuclear extract / labeled GAS probe complex. EMSA showed the binding of HaCaT nuclear extracts to GAS probe (a). Gel shift supershift analysis with specific IgG antibodies for STAT1. Nuclear extracts were preincubated with anti-STAT1 monoclonal antibodies for 3h at 4°C, prior to the addition of the labeled probe. The antibodies inhibited formation of the nuclear extract / labeled GAS probe complex (b).

antibody blocked formation of the nuclear extract / labeled GAS probe complex. In this cases, the antibody may specifically attach the STAT1 region of the nuclear extract and resulting in inhibition effect of the nuclear extract / probe complex formation (Fig. 2b).

## Discussion

STING is known as an IFN stimulator essential for host defense against RNA, DNA vi-ruses and intracellular bacteria, however, it was relatively unknown how cytokines affect the expression of STING. In this study, we demonstrated that the STING expression is regulated by stimulation of various cytokines



in human epidermal keratinocytes (HaCaT). Especially, IFN- $\gamma$  induced the STING mRNA expression.

IFNs are antiviral cytokines that are produced by many cell types following viral infection. IFNs are classified as either type I or type II. There are many type I IFNs, all of which have considerable structural homology. These include IFN- $\alpha$ , IFN- $\beta$ , IFN- $\delta$ , IFN- $\epsilon$ , IFN- $\kappa$ , IFN- $\tau$  and IFN- $\omega$ . IFN- $\alpha$ , IFN- $\beta$ , IFN- $\epsilon$ , IFN- $\kappa$  and IFN- $\omega$  exist in humans, whereas IFN- $\delta$  and IFN- $\tau$  have been described only for pigs and cattle, respectively, and do not have human homologues. By contrast, there is only one type II IFN, IFN- $\gamma$ . IFN- $\gamma$  is a markedly different cytokine than the type I IFNs, but it was originally classified in the IFN family because of its ability to 'interfere' with viral infections, which is consistent with the primary definition of IFNs<sup>6)</sup>. IFNs are very important regulator of JAK/STAT pathway. Binding of IFN- $\gamma$  to its receptor leads to tyrosine phosphorylation of the JAK1 and JAK2 tyrosine kinases, resulting in the phosphorylation of STAT1. Phosphorylated STAT1 homodimerizes to form the GAF-AAF complex, which translocates to the nucleus and binds to the GAS element present in most IFN- $\gamma$  inducible genes<sup>7, 8)</sup>. In this study, we showed that GAS element in STING promoter lesion is essential for IFN- $\gamma$ -regulated promoter activity, furthermore, it was revealed that STAT1 binds to the GAS element on the STING promoter region.

The present study provided the evidences that IFN- $\gamma$ /JAK/STAT1 signaling is critical for the expression of STING in human epidermal

keratinocytes.

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