Cellular Sensing of Viral DNA and Viral Evasion Mechanisms

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Abstract

Mammalian cells detect foreign DNA introduced as free DNA or as a result of microbial infection, leading to the induction of innate immune responses that block microbial replication and the activation of mechanisms that epigenetically silence the genes encoded by the foreign DNA. A number of DNA sensors localized to a variety of sites within the cell have been identified, and this review focuses on the mechanisms that detect viral DNA and how the resulting responses affect viral infections. Viruses have evolved mechanisms that inhibit these host sensors and signaling pathways, and the study of these antagonistic viral strategies has provided insight into the mechanisms of these host responses. The field of cellular sensing of foreign DNA is in its infancy, but our currently limited knowledge has raised a number of important questions for study.

Contents

INTRODUCTION

Microbial infections are initially detected by the host through cellular receptors that sense foreign microbial macromolecules. The recognition of pathogen-associated molecular patterns, or PAMPs, by the pathogen-recognition receptors, or PRRs, leads to activation of signaling pathways that induce innate immune responses that inhibit various stages of microbial growth. The PAMPs that have been best described are microbial proteins, lipids, carbohydrates, and RNA. It has been recently recognized that microbial DNA can also be sensed, leading to innate immune responses that help to control microbial infection. One of the key indicators of the importance of an immune response is defined by the microbe's attempt to evade or neutralize the innate immune response. Consistent with this, viruses have evolved elaborate mechanisms to hide their DNA from the cellular detection systems and/or inactivate the sensor or signaling mechanisms that are involved in these innate responses.

A number of excellent recent articles have reviewed mammalian DNA sensors and how they detect microbial DNAs and initiate innate responses (4, 58, 74). Therefore, we focus in this review on how these detection mechanisms integrate with viral replication mechanisms and how viruses have evolved to evade these sensing mechanisms. We examine how viral DNAs are revealed or hidden from the host cell to avoid setting off these danger alarms. Many of the signaling pathways for DNA sensing are just being worked out, and many questions and even paradoxes have arisen. We hope to delineate the major questions that need to be addressed in this area.

SIGNALING PATHWAYS ACTIVATED BY MICROBIAL DNA SENSING

The classical innate immunity pathways that are activated in response to viral DNA are induction of interferons (IFNs), cytokines, and proinflammatory cytokines (30). These effector molecules have direct antiviral activity, induce antiviral genes, and/or enhance the adaptive immune response to control viral infection. Recent studies have also shown that microbial DNA introduced by infection or foreign DNA introduced by transfection activates an additional cellular response resulting in the epigenetic silencing of the foreign DNA through a nuclear DNA sensor (56). We summarize each of these pathways in turn.

Expression of Cytokines and Interferons

The sensing of foreign DNA leads to the activation of several innate immunity pathways and the activation of an intrinsic resistance pathway. These are discussed individually in the following sections.

STING–IRF-3–interferon β. The induction of the antiviral type I IFN response is regulated by a cellular signaling cascade that results in the activation of IFN regulatory factor-3 (IRF-3) and nuclear factor–kappa B (NF-κB) transcription factors. A critical component of this signaling cascade is stimulator of IFN genes (STING) (29), an endoplasmic reticulum–transmembrane protein that bridges DNA-sensing mechanisms to downstream signaling events. Upon microbial DNA stimulation, STING relocalizes to distinct intracellular foci (66) and promotes TANK-binding kinase 1 (TBK1)-dependent phosphorylation of IRF-3 by a direct interaction with both proteins (73). Phosphorylated IRF-3 dimerizes, translocates to the nucleus, and binds to IFN-stimulated regulatory elements (ISRE) in the promoters of IRF-3-responsive genes, including type I IFNs. IFN is subsequently secreted from cells and can act in an autocrine or paracrine fashion to upregulate IFN-stimulated genes (ISGs) via the type I IFN receptor and a JAK-STAT signaling cascade (reviewed in 70). A subset of ISGs can also be induced independently of IFN, but IFN significantly amplifies this induction (78).

NF-κB. The NF-κB transcription factor also plays an important role in the expression of IFNs and cytokines in response to a variety of stimuli; however, the signaling cascade that activates NF-κB in response to microbial DNA is less well defined than that of IRF-3. Normally sequestered in the cytosol, NF-κB is released and enters the nucleus upon degradation of the regulatory IκB protein (reviewed in 54). Whereas STING has been shown to be crucial for the relocalization of NF-κB to the nucleus and subsequent expression of NF-κB-dependent genes (29), the mechanism by which STING activates NF-κB has not been elucidated.

Inflammasome signaling. Microbial DNA also activates an inflammatory response through the secretion of the IL-β and IL-18 proinflammatory cytokines (65). Whereas the expression of these genes is initially controlled at the transcriptional level by NF-κB activity, their release from the cell requires a posttranslational secondary signal mediated by a multiprotein complex known as the inflammasome. Recognition of DNA by cytosolic or nuclear DNA receptors results in the recruitment of the apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) adaptor protein and the subsequent autoproteolysis of procaspase 1 into active caspase 1. Caspase 1 then cleaves pro-IL-1β and pro-IL-18 into IL-1β and IL-18, respectively, which are then secreted from the cell as effector molecules.

Epigenetic Silencing of the Microbial DNA

In addition to activating innate immune responses, a host cell can respond to microbial or other foreign DNA through intrinsic mechanisms aimed at silencing foreign gene expression. This nuclear response involves the assembly of heterochromatin onto foreign DNA and has been observed during herpesvirus infection (40) and upon plasmid DNA transfection (11). Components of nuclear domain 10 (ND10) bodies, including PML, hDaxx, and Sp1, which are recruited to incoming viral DNA early during infection, have been implicated in this response owing to their ability to restrict viral gene expression (8), but the mechanisms by which this effect is exerted have

not been elucidated. As discussed below, the IFI16 DNA sensor has recently been shown to play a role in the epigenetic silencing of foreign DNA (56).

INTRACELLULAR SENSORS OF MICROBIAL DNA

Foreign DNA is detected as a danger signal when it is within the cell; therefore, foreign DNA sensors are located within the cell at various sites including vesicles in the cytoplasm, within the cytosol, or within the nucleus. As a consequence, the microbial DNA molecules that are sensed by mammalian cells are usually those of DNA viruses and intracellular bacteria, although there are examples of extracellular bacterial DNA activating intracellular sensors (79). The DNA viruses include those replicating in the cytoplasm, such as the poxviruses; those replicating in the nucleus, such as the herpesviruses; and the retroviruses. Retroviral reverse transcriptase converts the genomic RNA to double-stranded DNA (dsDNA) in the cytoplasm, and the genomic DNA copy is transported into the nucleus, where it is integrated into cellular chromosomes.

Membrane-Bound Receptors

The first DNA sensor identified was TLR9, a member of the Toll-like receptor (TLR) family of pattern-recognition receptors. Initially identified by sequence homology with previously identified TLR proteins (23), TLR9 is localized within the endosomal compartment and is membrane bound. Macrophages from TLR9$^{-/-}$ mice showed a reduction in inflammatory cytokine production in response to unmethylated CpG DNA (23). Furthermore, TLR9$^{-/-}$ mice have been shown to be more susceptible to bacterial infections like *Mycobacterium tuberculosis* (7) and viral infections like herpes simplex virus (HSV) (69). TLR9 expression is cell-type restricted and mainly observed in immune cells, including plasmacytoid dendritic cells (pDCs) and B cells. Although pDCs are potent cytokine producers and play a major role in controlling the systemic spread of pathogens like HSV (37), they are not likely to be the first cells to come in contact with a pathogen in vivo; thus, TLR9 may play only a limited role in the antiviral response at the primary site of infection. Indeed, HSV-1 can replicate in the cornea as efficiently in the presence of TLR9 as in its absence (42), whereas type I IFN receptors are critical for the control of viral infection in this tissue (14).

The previous study (14) highlights the importance of TLR9-independent mechanisms in controlling virus replication in vivo, and other studies have revealed that nonimmune cells (e.g., fibroblasts and epithelial cells), which do not express TLR9, can induce DNA-dependent immune responses, indicating that additional sensing mechanisms exist. Furthermore, TLR9-deficient dendritic cells and monocytes can still respond to transfected viral DNA (28) or DNA virus infection (24), suggesting that innate immune cells possess multiple mechanisms of DNA sensing.

Cytosolic Sensors

There are several proteins implicated as cytosolic sensors of foreign DNA, and they will be discussed individually in the following sections.

DNA-dependent activator of interferon. The field of cytosolic DNA sensing began with the identification of the DNA-dependent activator of IFN (DAI) protein. Exogenous expression of DAI in L929 mouse cells resulted in increased cytokine expression in response to transfected B-form DNA, and small interfering RNA (siRNA) depletion of DAI decreased the IFN response to HSV-1 (72). These observations along with the protein's DNA binding activity led to its classification as a putative DNA sensor. However, subsequent studies revealed redundancy to

DAI-dependent antiviral cytokine expression as $DAI^{-/-}$ cells and mice responded normally to immunostimulatory DNA (28). Interestingly, DAI appears to have an effect on HSV infection independent of its putative DNA-sensing activity. DAI depletion resulted in enhanced HSV-1 replication due to increased expression of the viral ICP0 protein (61), an antagonist of cellular DNA sensing (57). Therefore, it is possible that the enhanced antiviral response to HSV infection observed by Takaoka et al. (72) in the absence of DAI is a result of enhanced inhibition of sensing by the virus itself.

Recently, DAI has also been implicated as a critical component of the receptor interacting protein 3 (RIP3)-dependent necrosis pathway in response to mouse cytomegalovirus (mCMV) infection (76). This nonredundant role of DAI was revealed only upon infection with an mCMV mutant virus that lacked an antagonist of the DAI-RIP3 pathway, highlighting the importance of using the appropriate stimuli to investigate the activity of a cellular protein. Wild-type (WT) mCMV was able to replicate efficiently in both $DAI^{-/-}$ and $DAI^{+/+}$ mice, whereas a viral inhibitor of RIP activation (vIRA) mutant was unable to establish a productive infection in WT mice but replicated normally in the absence of DAI. In the same study, vIRA was shown to disrupt DAI binding to RIP3, defining the mechanism of inhibition used by the virus. Whether this novel activity of DAI is dependent on its DNA-binding activity has not been determined and remains an important experiment to link DNA sensing to DAI-dependent necrosis.

RNA polymerase III. In 2009, two groups reported the identification of a DNA-sensing pathway that signaled through a previously defined RNA-sensing mechanism (3, 12). RNA polymerase (pol) III was demonstrated to transcribe AT-rich DNA to produce 5′ triphosphate RNA molecules that can be recognized by the RIG-I signaling system. However, the activity of this protein in sensing microbial infection remains controversial. Although RNA pol III was initially reported to be involved in sensing both HSV DNA and *Legionella pneumophila* infection (12), others have observed that IFN production in response to these pathogens is independent of RNA pol III activity (47, 48, 75). Other herpesviruses, like Epstein-Barr virus, encode genes (e.g., EBER genes) that are normally transcribed by RNA pol III (27), indicating that RNA pol III may have a more substantial role in the life cycle of specific pathogens. Indeed, treatment of EBV-positive Burkitt lymphoma cells with an RNA pol III inhibitor resulted in decreased IFN production (3). Interestingly, the RNA pol III pathway appears to be functional in a variety of tumor cell lines where other DNA-dependent pathways (STING-mediated) appear to be dysfunctional (e.g., HEK293T cells). Therefore, RNA pol III may be redundant in cells where other DNA-sensing mechanisms are functional.

PYHIN family proteins. In addition to activating IRF-3- and NF-κB-dependent signaling cascades, cytosolic DNA can activate an ASC-dependent inflammasome-mediated response resulting in the secretion of proinflammatory cytokines (53). A DNA-binding protein that plays a major role in this response is absent in melanoma 2 (AIM2), a member of the PYHIN (pyrin and HIN-containing) protein family (19, 26). Bacterial and viral infection (vaccinia virus and mCMV) of AIM2-deficient cells resulted in decreased secretion of proinflammatory cytokines compared to WT cells (34, 64), and AIM2-deficient mice are more susceptible to infection with the cytosolic bacterium *Francisella tularensis* (19), confirming the importance of this protein in vivo. Interestingly, whereas mouse AIM2 appears to be strictly involved in inflammasome signaling, exogenous expression of human AIM2 induces both proinflammatory cytokine secretion and ISRE-dependent responses, suggesting these proteins may not be true functional orthologs (9). Furthermore, there appears to be considerable diversity in mice with regards to PYHIN proteins; whereas human

genomes encode only 4 members of this family, 14 mouse proteins have been identified with varying degrees of functional redundancy (9).

Another member of the PYHIN family of proteins, IFN-inducible protein 16 (IFI16), has been described as a microbial DNA sensor involved in IRF-3-mediated signaling. IFI16 was identified during a screen of proteins from cytosolic extracts of THP1 cells that copurified with a biotin-labeled immunostimulatory vaccinia virus DNA motif. Depletion of the mouse functional ortholog of IFI16, p204, resulted in decreased IRF-3 and NF-κB responses to HSV infection in mouse macrophages (75). The ability of IFI16 to promote IRF-3 signaling in response to HSV was later confirmed in primary human fibroblasts (57) and primary human macrophages (25). IFI16 also appears to play a role in inflammasome signaling and can associate with the ASC adaptor protein, particularly in response to infection with gammaherpesviruses (6, 38, 68). Most recently, IFI16 has also been implicated in the cellular response to lentivirus infection and has been found to recognize single-stranded DNA with secondary structure that mimics dsDNA (31).

Like AIM2, IFI16 appears to sense DNA through sequence-independent contacts with the sugar phosphate backbone (32), which is consistent with the sequence-independent nature of these sensing molecules. However, unlike AIM2, which is strictly localized to the cytoplasm, IFI16 can be found in both the nucleus and the cytoplasm in a cell type–dependent manner (77). This pattern of localization, which is controlled at least in part by the acetylation of the IFI16 nuclear localization signal (44), has been proposed to broaden the ability of the cell to detect viruses that replicate in the nucleus (see below).

Because mouse genomes encode numerous PYHIN family proteins, it is difficult to define the precise homolog of IFI16 in mice. The murine p204 gene product appears to be the functional homolog of IFI16 in mice, but this remains to be formally proven. To date, p204-deficient mice have not been described. Depletion of p204 expression in the corneas of mice led to increased HSV-1 replication in the corneal tissue (14). Therefore, p204 and by extrapolation IFI16 may have a role in controlling HSV-1 replication in vivo.

Human genomes encode two additional members of the PYHIN family, IFIX and MNDA, and overexpression experiments suggest these proteins may also be involved in innate immune signaling responses (9); however, their activities during infection have not been formally examined.

DExD/H-box helicases. Prompted by the observation that certain populations of TLR9-deficient dendritic cells could still activate an IFN response to DNA virus infection (24), several labs screened for additional DNA-sensing mechanisms in these cells. A proteomics screen for proteins binding to CpG-containing DNA identified two members of the DExD/H-box helicase superfamily, DHX36 and DHX9, as putative DNA sensors in pDCs (39). Depletion of these two proteins individually by siRNA resulted in differential effects on proinflammatory cytokine and IFN production in response to DNA virus infection. Whereas DHX36 depletion decreased IFN-α but not TNF-α production in response to HSV-1, the opposite was true for DHX9. This phenotype corresponded with differential activity of these two proteins in inducing IRF-7 and p50 nuclear accumulation. However, both proteins interacted with the MYD88 adaptor protein, which was required for activation of both IRF-7 and p50; therefore, it is still unclear how these proteins differentially activate downstream signaling cascades.

The DExD/H-box helicase superfamily contains a total of 59 proteins, including members of the RIG-I-like family of helicases, suggesting that additional family members may be involved in intracellular nucleic acid sensing. Indeed, screening of additional DExD/H-box helicase proteins resulted in the identification of DDX41 as an important component in microbial DNA sensing (83). Depletion of DDX41 resulted in decreased IFN-a/β secretion in response to HSV, adenovirus, and *Listeria monocytogenes*. The activity of DDX41 was not limited to myeloid dendritic cells,

as other innate immune cells (e.g., monocytes) also showed a dependence on DDX41 for their response to microbial infection.

Although the original activity of DDX41 was thought to be dependent on direct DNA-binding activity (83), DDX41 has also been reported to bind to the bacterial secondary messengers cyclic di-AMP and cyclic di-GMP (59). These dinucleotides are potent activators of cytokines and IFN (46, 80) and likely play a role in the activation of these responses to bacterial infection. Unlike bacteria, viruses have not been shown to produce their own dinucleotides; however, the recent identification of DNA-dependent cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) production (81) by mammalian cGAMP synthase (cGAS) (71) during virus infection (discussed in detail below) raises the question of whether the activity of DDX41 can be extended to cGAMP sensing and whether this could account for the DDX41-dependent response to DNA viruses. Furthermore, it is currently unclear whether DDX41 merely enhances STING-dependent cyclic-dinucleotide binding, as depletion of DDX41 in 293T cells reduced dinucleotide binding to ectopically expressed STING (59). Therefore, it is perhaps too early to characterize DDX41 as a bona fide DNA sensor, although it clearly plays an important role in microbial DNA sensing.

DNA-dependent protein kinase. In another screen of cytosolic DNA-interacting proteins, Ferguson et al. (18) identified DNA-dependent protein kinase (DNA-PK) as a DNA sensor. This protein has a well-defined role in nuclear DNA damage responses, including nonhomologous end-joining (NHEJ) (10), but a role for cytoplasmic DNA-PK has not been described. IRF-3 signaling was reduced in DNA-PK$^{-/-}$ mouse fibroblasts and in vivo in response to transfected immunostimulatory DNA (ISD) or vaccinia virus infection. This activity of DNA-PK was independent of its protein kinase activity, suggesting that the previously described DNA-PK-dependent phosphorylation of IRF-3 (36) was not necessary for activation under the reported conditions. In addition, the authors observed no change in DNA-dependent NF-κB activation in DNA-PK$^{-/-}$ mouse fibroblasts, indicating additional sensing mechanisms are active. The discovery of a vaccinia virus protein that inhibited the innate signaling capability of DNA-PK greatly strengthens the argument for the involvement of DNA-PK in DNA sensing. Mice infected with a C16 deletion mutant virus produced higher levels of cytokines than mice infected with a WT virus, further confirming the importance of this signaling pathway in innate immunity (60).

The exact mechanism by which DNA-PK perpetuates downstream signaling is unclear. The Ku70 component of DNA-PK was observed to coimmunoprecipitate with STING in unstimulated HEK293 cells, and this association was lost upon DNA stimulation (18). However, whether this release of STING is necessary for the activation of downstream IRF-3 has not been resolved.

cGAS. Recently, cGAS was identified as a cytosolic DNA sensor (71). In response to DNA, cGAS produces the cyclic dinucleotide cGAMP (81), which binds to and activates STING-dependent IFN production. Cells from mice deficient in cGAS show reduced antiviral responses, and mice are more susceptible to DNA virus infection in the absence of cGAS (45). cGAS has also been implicated in the response to retroviruses like HIV (20, 31), whose viral RNA genome is converted to DNA through a reverse transcription mechanism. Surprisingly, a recent study demonstrated that cGAS$^{-/-}$ mice were also more susceptible to RNA virus infection (67), indicating that cGAS activity may be pan-antiviral. In addition, Schoggins et al. (67) observed a reduction in the basal levels of IFN-β and several ISGs in cGAS-deficient bone marrow–derived macrophages, implicating cGAS in the basal homeostasis of innate signaling. cGAMP can also spread to neighboring cells via gap junctions to activate STING (5). Many proposed DNA sensors, including IFI16, are IFN inducible; therefore, cGAS depletion could indirectly affect the ability of additional DNA

sensors to induce antiviral cytokine production. Currently no microbial gene products have been identified that specifically inhibit the activity of cGAS.

STING. STING has long been recognized as a critical signaling molecule that links DNA sensors to IRF-3. Moreover, a recent paper reported that STING binds directly to DNA in vitro and in vivo and may directly activate the innate signaling pathway itself (1). Further work is needed to show that STING directly senses foreign DNA without the aid of other DNA sensors, but this is a potential additional DNA-sensing molecule.

VIRAL REPLICATION AND CELLULAR DNA SENSING

Sensing of Herpesviral DNA

Herpesviruses enter cells by fusion at the plasma membrane or via endocytosis and fusion in the vesicles. In permissive cells such as keratinocytes and fibroblasts, the released nucleocapsid is transported via microtubules to the nuclear pore where the nucleocapsid docks, and the viral dsDNA genome is released into the nucleus. By the conventional infection pathway (**Figure 1**), this is the first stage at which the herpesviral DNA is available for binding by host cell factors. The viral DNA is not associated with histones or viral proteins, and so it is initially not organized in chromatin in the cell nucleus, but histones are rapidly assembled on the viral genome (13, 55). Kaposi sarcoma–associated herpesvirus infection was first shown to induce inflammasome responses in the cytoplasm by nuclear IFI16 recognizing viral DNA in the nucleus and moving to the cytoplasm to promote the assembly of inflammasomes (38). IFI16-dependent activation of inflammasome signaling has also been demonstrated in human fibroblasts during HSV-1 infection (33).

Wild-type HSV-1 induces minimal IFN-β, but when viral protein synthesis is inhibited type I IFNs are induced (52, 62), supporting the idea that a viral protein inhibits IFN induction. Viral mutants defective for ICP0 induce the highest level of IFN-β (17, 57). In normal human fibroblasts HSV-1 recombinant strains that do not express ICP0 induce IRF-3 signaling in an IFI16-dependent manner (57). Viral DNA must be delivered to the nucleus (57) and IFI16 must be localized to the nucleus (44) for IFI16-induced IFN-β induction. This is consistent with the infection pathway in which the viral DNA is exposed to the cell only when it is released into the cell nucleus (**Figure 1**).

Other studies have shown that cytosolic cGAS is required for IRF-3 dimerization in response to HSV-1 Δ34.5 virus infection of THP-1 cells (71). Further studies of *cgas*$^{-/-}$ murine lung fibroblasts and bone marrow–derived dendritic cells showed that cGAS is required for IFN-β induction by WT HSV-1, HSV-1 7134 ICP0-null virus, or HSV-1 *d*109 mutant virus (45). These results supported the idea that cGAS is required for IFN induction by HSV-1 in murine cells. Surprisingly, cGAS was also required for IFN induction in *cgas*$^{-/-}$ versus *cgas*$^{+/+}$ plasmacytoid dendritic cells upon infection with WT HSV-1 (45). This result supported the idea that there is some cross talk between cGAS and TLR9 and this cross talk is involved in the major IFN-α response in pDCs.

Therefore, we currently have a paradox in which nuclear IFI16 is required for IRF-3 signaling in some cell types whereas cytosolic cGAS is required for IRF-3 signaling in other cell types. The important question is whether both are required in the same cell types. Our own results show that both IFI16 and cGAS are required for IRF-3 signaling in human foreskin fibroblasts (M.H. Orzalli, N.M. Broekema, and D.M. Knipe, manuscript in preparation). These results could mean that IFI16 and cGAS work together or that their signaling pathways interact in some way, but clearly more studies are needed to resolve this important issue.

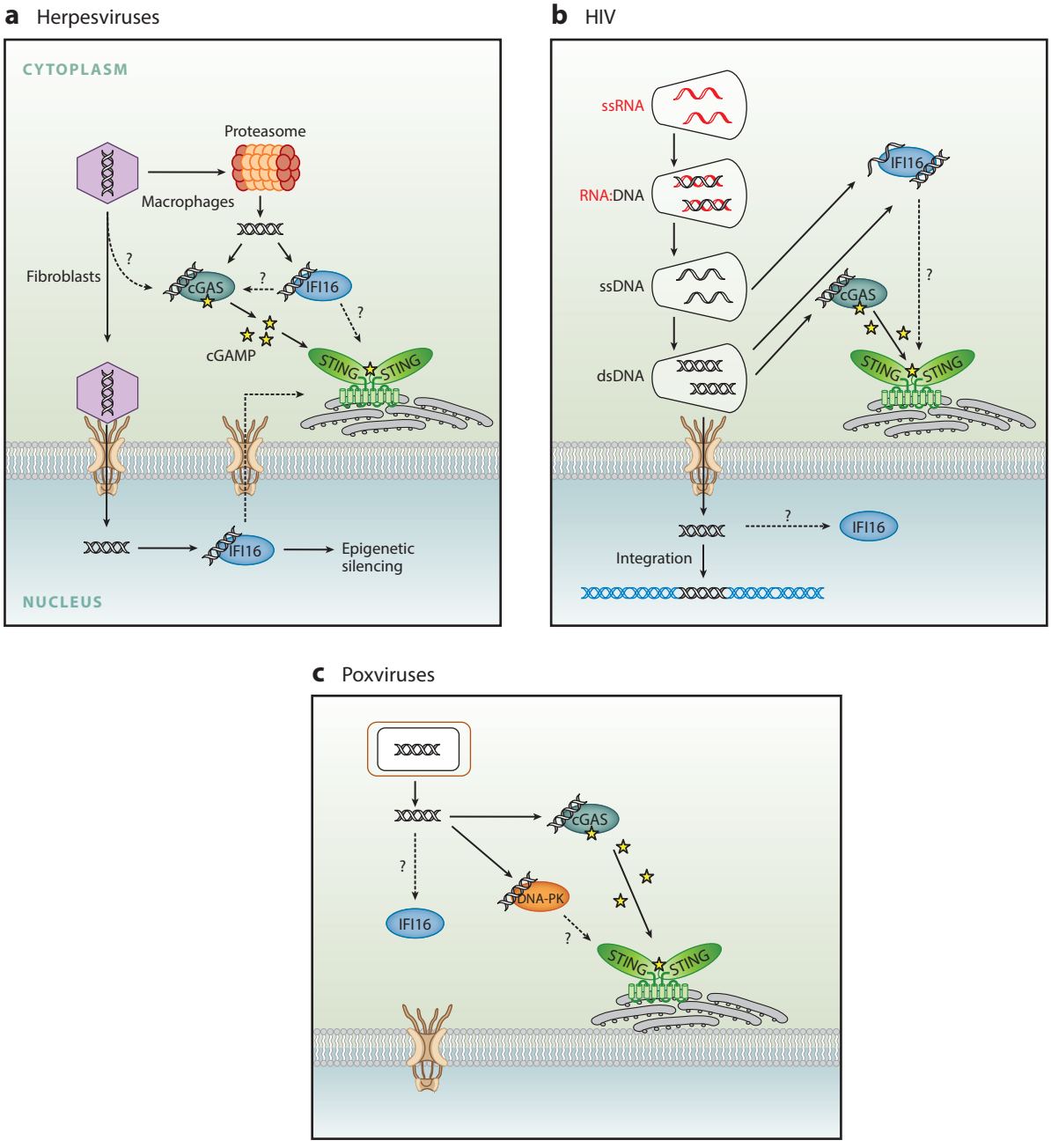

Figure 1

Models of viral DNA sensing. (*a*) Herpesviruses: Upon entry into cells, viral DNA is released into either the nucleus (fibroblasts) or cytosol (macrophages). IFI16 or cGAS senses the viral DNA and activates downstream signaling pathways. (*b*) HIV: Upon entry into the cytoplasm, viral RNA undergoes reverse transcription to produce single-stranded DNA (ssDNA) intermediates or final double-stranded DNA (dsDNA) molecules that are sensed by cytosolic sensors. Viral DNA enters the nucleus and integrates into cellular chromatin. (*c*) Poxviruses: Release of viral DNA into the cytosol activates DNA-PK and/or cGAS-dependent signaling. Other abbreviations: IFI16, interferon-inducible protein 16; cGAMP, cyclic GAMP; cGAS, cyclic GAMP synthase; DNA-PK, DNA-dependent protein kinase; STING, stimulator of interferon genes. Dashed arrows depict an undefined mechanism or a process that has not been examined.

In contrast to fibroblasts, macrophages possess a mechanism that releases viral DNA into the cytosol, where it can then be sensed by cytosolic proteins. This response, reported by Horan et al. (25), apparently involves the proteasomal degradation of the viral capsid as it transits through the cytoplasm to the nuclear pore. Although these authors conclude that this system is not functional in fibroblasts, the exact cellular components that target the viral capsid for degradation have not been defined. Importantly, IFI16 is localized in the cytoplasm in macrophages, and this is required for IFN induction in response to viral infection (25). This mechanism may explain the relative nonpermissivity of human macrophages to HSV replication, as the viral DNA aberrantly accumulates in the cytoplasm; furthermore, this may represent a general difference in sensing location for cells permissive and nonpermissive to herpesvirus infection.

There is increasing evidence that IFI16 plays an inhibitory role in the replication of herpesviruses independent of its ability to activate IRF-3 and inflammasome signaling pathways. Gariano et al. (21) reported that IFI16 restricts human cytomegalovirus (HCMV) early gene expression and subsequent DNA replication by sequestering the SP1 transcription factor away from the viral genome (21). In contrast, others have reported a positive role for IFI16 in HCMV replication (15); therefore, IFI16 activity during CMV infection is still unclear. Interestingly, we have observed that IFI16 restricts HSV-1 replication at the stage of immediate early gene expression by inducing heterochromatin formation on the viral genome in the nucleus (56), supporting the idea that IFI16 plays a negative role in herpesvirus replication.

The evidence for nuclear sensing of viral DNA raises the question of how this sensor distinguishes between foreign and self DNA. This question has yet to be answered, but there are some intriguing hypotheses and results starting to accumulate. We hypothesized in our original paper on IFI16 sensing that the difference in specificity might be due to the underchromatinization or loose chromatin on the viral genome (57). Consistent with this, our studies on IFI16 promoting epigenetic regulation showed that IFI16-dependent restriction occurred on transfected SV40 DNA, which is not chromatinized, but did not occur on SV40 DNA delivered by the virion (56). A recent article showed that IFI16 binds cooperatively to dsDNA 33 base pairs or larger and does not bind efficiently to DNA the size of the linker DNA between nucleosomes or at a transcription bubble (50). Those authors also speculated that IFI16 would not bind efficiently to self DNA assembled in nucleosomes.

Herpesviral evasion of DNA sensing. Viruses often target key points in host cell metabolism to manipulate the cellular processes to their own advantage. The importance of a host molecule in resisting viral infection can sometimes be estimated by the number of viral gene products or mechanisms that act to counter the functions of the host gene product. In this regard, there are two herpesviral proteins, HSV-1 ICP0 and HCMV pUL83, that target IFI16, but none are yet known to target cGAS.

HSV-1 ICP0 promotes the degradation of IFI16 in normal human fibroblasts (33, 57). The reduction in IFI16 requires the RING domain of ICP0 and proteasomal activity of the host cell (57), supporting the idea that the loss of IFI16 involves the ubiquitination activity of ICP0 and proteasomal degradation. It remains to be determined if this involves a direct interaction between ICP0 and IFI16 and whether IFI16 is a target of ICP0's E3 ubiquitin ligase activity. It has been reported that ICP0 is not necessary for degradation of IFI16 in HSV-1-infected cells (16), but these studies largely involved HepaRG and U2OS tumor cells. The lack of degradation of IFI16 during HSV-1 infection of tumor cells has been observed by others (56). IFI16 is often not expressed or nonfunctional in tumor cells (44, 56), so IFI16 may need to be bound to DNA or otherwise activated for ICP0-promoted degradation to occur. There is indeed a modest reduction in IFI16

in normal fibroblasts infected with *ICP0*-null mutant viruses (57), but it is much less than when ICP0 is expressed.

HCMV evasion of DNA sensing. HCMV pUL83 inhibits IFI16-mediated innate signaling by binding to the pyrin domain of IFI16 and blocking the multimerization of IFI16 when bound to DNA (43). pUL83 is a major component of the viral tegument and can mediate its inhibitory activity prior to the expression of other viral immunomodulatory proteins. Interestingly, whereas transfected UL83 inhibits filament formation of exogenously expressed pyrin domains in the absence of viral infection, IFI16 filaments have not been observed during *UL83*-null virus infection. This is in contrast to HSV-1 infection where IFI16 filament formation is observed in the absence of ICP0 (56) and could indicate that HCMV encodes an additional protein that inhibits IFI16 activity.

Sensing of Lentiviral DNA

HIV enters the host cell by fusion at the plasma membrane releasing the viral core particle into the cytoplasm. This complex migrates to microtubules, where reverse transcription occurs. Reverse transcription involves copying of the viral genomic RNA by the reverse transcriptase to yield a cDNA copy, which is partially single stranded. The preinitiation complex is then transported into the nucleus, where integration of the viral DNA into the host chromosome is catalyzed by the viral integrase enzyme. Minimal IRF-3 signaling is induced by HIV infection in murine cells, unless the host TREX1 endonuclease is defective (82). This was interpreted to mean that the reverse transcriptase products that are exposed to cellular sensors are normally degraded by the TREX1 endonuclease. A recent article showed that capsid protein mutations lead to increased IFN-β induction and reduced viral replication (63). The capsid protein mutations also result in decreased recruitment of host factor CPSF6 and cyclophilins Nup358 and CypA. Depletion of CPSF6 also led to increased IFN-β induction, supporting the idea that decreased capsid stability, decreased recruitment of host factors, or both lead to increased sensing of HIV-1 DNA.

In terms of the sensor that detects HIV-1 DNA, there is evidence that both cGAS and IFI16 can sense HIV-1 DNA. Gao et al. (20) found that in HIV-GFP-infected THP1 cells, reverse transcription is required for induction of IFN-β but integration of the viral DNA is not required. cGAS was required for IFN-β induction, and HIV infection induced production of cGAMP. TREX1 depletion was required for signaling in murine L929 cells but apparently not in human THP1 cells. Cytosolic extracts from HIV-GFP infection of HEK293T cells incubated with purified cGAS induced cGAMP, showing that the cytoplasm contains DNA capable of activating cGAS.

In contrast, Jakobsen et al. (31) reported that IFI16 colocalized and associated with lentiviral DNA in the cytoplasm of human macrophages and was required for activation of the STING-TBK1-IRF-3/7 innate response pathway. Depletion of IFI16 by short hairpin RNA (shRNA) knockdown in THP1 cells augmented lentiviral transduction and HIV-1 replication. In fact, knockdown of IFI16, cGAS, or STING increased HIV-1 replication, indicating that IFI16 and cGAS may both be necessary to decrease viral replication.

Quiescent $CD4^+$ T cells are not permissive for HIV replication but undergo abortive infection that results in incomplete DNA transcripts. These cytosolic viral DNAs are sensed and activate a form of programmed cell death called pyroptosis. Monroe et al. (49) recently reported that IFI16 was necessary for this CD4 T cell death. This raises the possibility of another pathway that is activated by sensing of foreign DNA.

Therefore, the situation with HIV is similar to that for the herpesviruses. Both IFI16 and cGAS have been reported to be required for sensing of these viral DNAs. Further studies to

clarify whether these two molecules function in a common innate sensing pathway or in two interconnecting pathways in one cell are of the highest priority.

Sensing of Poxviral DNA

Poxviruses enter the host cell by surface fusion or endocytosis, releasing the viral core into the cytoplasm (51). The viral core contains a complete transcriptional apparatus that synthesizes viral early mRNAs. Early protein products further uncoat the genome and promote viral DNA replication in the cytoplasm. The uncoated progeny DNA and/or the progeny viral DNA could be sensed by cytosolic DNA sensors. A recent article identified DNA-PK as essential for IRF-3 signaling in response to vaccinia virus infection (18). However, the vaccinia virus C16 protein product binds to the Ku dimer portion of the DNA-PK enzyme complex and inhibits this activity (60).

Vaccinia virus infection is also reported to activate cGAS in human HEK293T cells to produce cGAMP that can spread through gap junctions to activate STING in adjacent cells (5). Therefore, both DNA-PK and cGAS may be able to sense vaccinia and other poxviral DNAs, and further studies are needed to define their relative roles and importance in different cell types.

CONCLUSIONS AND CLOSING REMARKS

Cells possess mechanisms to detect foreign DNA introduced by transfection or infection with DNA viruses, and the sensing of this foreign DNA elicits innate responses and intrinsic resistance mechanisms. This is a recent area of study, and only the basic outlines of the molecules and mechanisms involved have been defined. Importantly, a large number of questions have arisen from our limited knowledge of this area.

Why is there such a multiplicity of sensors? The large number of potential DNA sensors has already been noted (74). As described previously, the potential receptors may not all be true DNA-sensing molecules, some may be working together, different cell types may have different sensors, and there may be redundancy in their activity. As discussed above, IFI16 and cGAS are both required for DNA sensing of HSV and HIV and therefore may be acting together or functioning in pathways that converge at some downstream signaling component.

Are these sensing mechanisms operating on other DNA viruses? Do other DNA viruses have evasion mechanisms? Herpesviruses, particularly HSV-1, have become the model DNA viruses used to study DNA sensing mechanisms; however, it is unclear whether the defined responses to HSV-1 can be extended to other DNA viruses. This is partly due to the lack of information about the DNA-dependent innate immune responses to other nuclear replicating viruses, including hepadnaviruses, papillomaviruses, and polyomavirus family members. Like herpesviruses, these DNA viruses likely encode proteins that inhibit innate immune signaling or have mechanisms to shield their DNA from detection. Indeed, a study examining the upregulation of cellular genes during BK polyomavirus infection of primary kidney epithelial cells found no increase in cellular cytokine mRNAs (2), suggesting that BK virus may evade the host innate immune response. The determination of whether these viruses are sensed by DNA-dependent mechanisms or whether they target specific signaling components to evade the host response will broaden our understanding of the role for DNA responses in virus infection.

Why are sensors located at different sites in different cell types? How is the intracellular site of a sensor regulated? The differential placement of sensors within diverse compartments of the cell provides mechanisms for sentinel surveillance that correspond with the various routes of entry used by viruses (22) and the sites where they replicate their genomic material (35). Similarly, the

ability of the cellular location of these sensors to be regulated may provide versatility in their ability to optimally sense foreign DNA. IFI16 appears to be the clearest example of this, with its cytoplasmic or nuclear localization dependent on the cell type examined. It will be interesting to determine whether other DNA sensors share similar cell type–dependent localization patterns.

What is their role in vivo? This is ultimately the most important question but will be difficult to resolve, given the apparent multiplicity and possible redundancy in sensing molecules and mechanisms. Nevertheless, advances in our knowledge of these sensors and their signaling mechanisms should allow the individual inactivation of these molecules to define their role in vivo.

The cellular molecules that sense foreign DNA are important for cellular innate responses and intrinsic resistance to DNA virus infection. The definition of their specific roles and mechanisms of action are likely to be important for designing new strategies for preventing and treating DNA virus infections. This information will also provide important knowledge about host innate immune response mechanisms. Furthermore, these molecules are likely to have important roles in cellular homeostasis, as evidenced by the defective nature of IFI16 in nearly all cancer cells and the reduction in basal ISGs in cGAS$^{-/-}$ cells. Thus, the study of these DNA-sensing molecules and signaling pathways should also yield important information about homeostatic and growth mechanisms in the host cell.

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