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Multiple signaling pathways leading to the activation of interferon regulatory factor 3

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Abstract

Virus infection of susceptible cells activates multiple signaling pathways that orchestrate the activation of genes, such as cytokines, involved in the antiviral and innate immune response. Among the kinases induced are the mitogen-activated protein (MAP) kinases, Jun-amino terminal kinases (JNK) and p38, the IκB kinase (IKK) and DNA-PK. In addition, virus infection also activates an uncharacterized VAK responsible for the C-terminal phosphorylation and subsequent activation of interferon regulatory factor 3 (IRF-3). Virus-mediated activation of IRF-3 through VAK is dependent on viral entry and transcription, since replication deficient virus failed to induce IRF-3 activity. The pathways leading to VAK activation are not well characterized, but IRF-3 appears to represent a novel cellular detection pathway that recognizes viral nucleocapsid (N) structure. Recently, the range of inducers responsible for IRF-3 activation has increased. In addition to virus infection, recognition of bacterial infection mediated through lipopolysaccharide by Toll-like receptor 4 has also been reported. Furthermore, MAP kinase kinase kinase (MAP KKK)-related pathways and DNA-PK induce N-terminal phosphorylation of IRF-3. This review summarizes recent observations in the identification of novel signaling pathways leading to IRF-3 activation.

Keywords: Interferon; Transcription; IRF-3; Toll-like receptors

1. Introduction

The success of the innate host defense to viral and bacterial infections is dependent on the ability of the cell to detect the presence of the invading pathogen. In response to the recognition of components specific to viruses and bacteria, such as constituents of the bacterial wall, the viral envelop, bacterial DNA or double stranded RNA (dsRNA; a by-product of viral replication), the host cell activates a multitude of signal transduction cascades that produce protein messengers in the form of cytokines, including interferons (IFNs), interleukins (IL), and tumor necrosis factor (TNF) that impede viral/bacterial replication and spread through innate and adaptive immune mechanisms [1,2]. Arguably, the best-characterized component of the innate host defense to virus is the family of transcriptionally activated IFN proteins, which includes type I IFN-α, IFN-β and type II IFN-γ. The induction of type I IFNs is mainly attributed to infection by various types of RNA and DNA viruses [2,3], although the bacterial endotoxin lipopolysaccharide (LPS) induces production of IFN in certain cells, albeit at low levels [4,5]. Once produced, these

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Abbreviations: IFN, interferon; IRF, IFN regulatory factor; ISRE, IFN-stimulated regulatory element; NF-κB, nuclear factor kappa B; IκBα, inhibitor kappa B alpha; ISG, interferon stimulated gene; STAT, signal transducer and activator of transcription; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; IRAK, IL-1R-associated kinase; TRAF, TNFR-associated factor; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; CBP, CREB-binding protein; IP-10, IFN-γ-inducible protein 10; ISGF3, interferon stimulated gene factor 3; TNF, tumor necrosis factor; IL, interleukin; dsRNA, double stranded RNA; PKR, dsRNA activated kinase; MHC, major histocompatibility complex; MAL, MyD88 adapter-like; AP-1, activator protein-1; IKK, IκB kinase.

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secreted proteins act in a paracrine fashion to induce gene expression in target cells in the adjacent microenvironment, through engagement of cell surface IFN receptors and the JAK-STAT signaling pathway. STAT1/2 heterodimers, in conjunction with interferon-stimulated gene factor 3γ (ISGF3γ/IRF-9) bind to ISRE found in numerous IFN-induced genes, such as 2′–5′ oligoadenylate synthase and the dsRNA activated kinase (PKR), resulting in the induction of proteins which impair viral gene expression and replication [2,3]. In addition to their antiviral activity, IFNs have other pleiotropic effects in the host, with important roles in apoptosis, growth inhibition, and development of protective immune responses *via* increased expression of MHC class I proteins [6] and other components of adaptive immunity. IFNs thus link the innate immune responses to adaptive immunity [7].

2. Multiple transcription factors are activated following virus infection

The rapid induction of type I IFNs following virus infection requires the posttranslational modification of latent transcription factors involved in immunomodulation. Multiple distinct signaling pathways are activated by virus infection, leading to phosphorylation events that induce the activation of NF-κB [8], interferon regulatory factor 3 (IRF-3) [9–14], and ATF-2/c-Jun (AP-1) [15] and activation of specific target genes (Fig. 1). The transduction pathways involved in NF-κB and ATF-2/c-Jun activation have been well characterized. Virus infection or dsRNA treatment leads to the activation of the stress-activated members of the MAP kinase (MAP K) superfamily, namely the c-Jun amino terminal kinases (JNKs) and p38 proteins which are

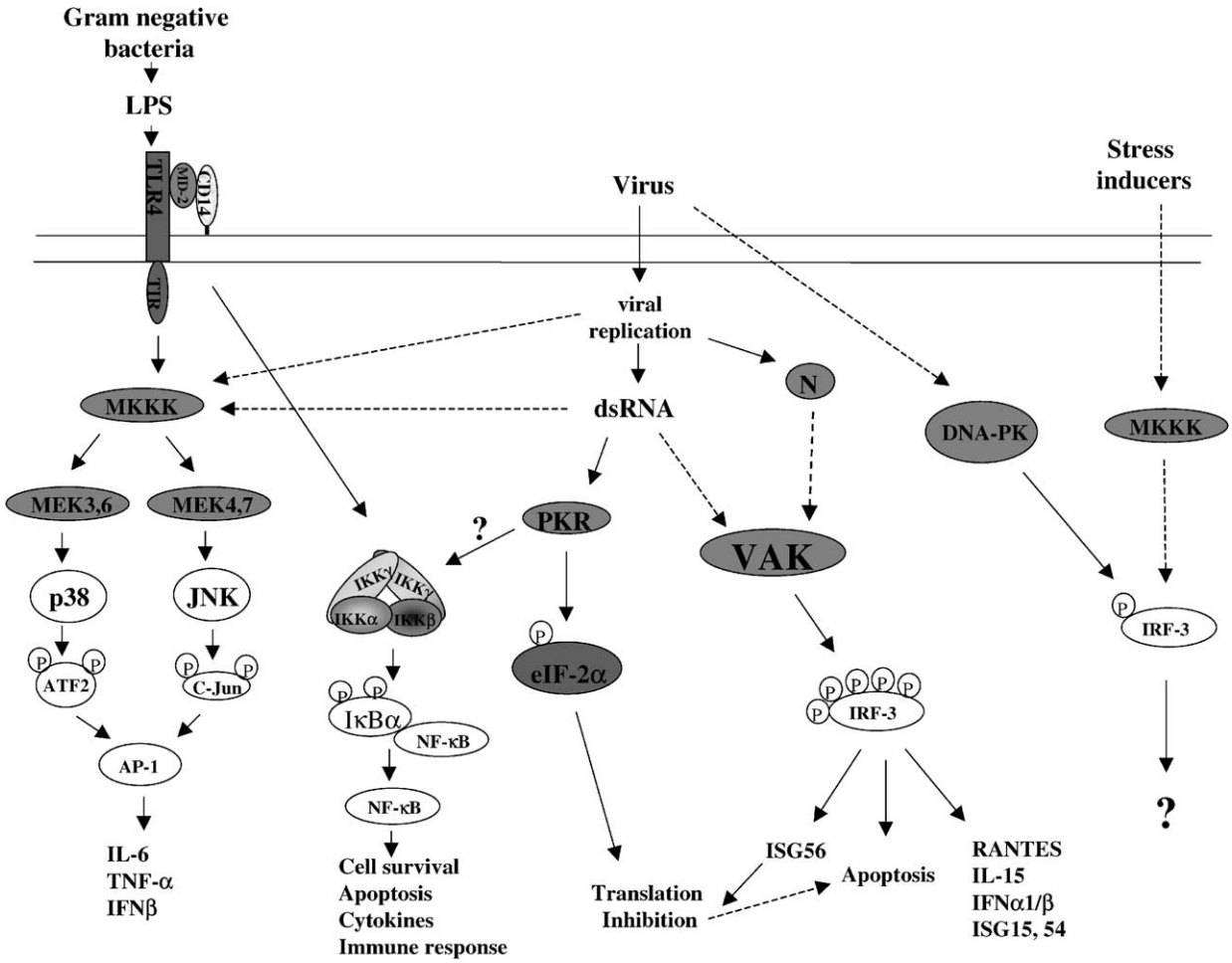

Fig. 1. A model of signaling cascades triggered by viruses and PAMPs. Virus infection results in the activation of multiple signaling cascades resulting in the phosphorylation of ATF-2, C-Jun, IRF-3, and NF-κB. Following virus replication, the generation of dsRNA activates the stress-induced MAPK pathway p38/JNK resulting in the AP-1 activation. PKR is activated following binding to dsRNA, phosphorylates eIF-2α, and inhibits protein translation. PKR may also associate with the IKKβ subunit. Viral nucleocapsid (N) and dsRNA activate VAK, a virus activated kinase, leading to C-terminal phosphorylation of IRF-3. IRF-3 activation stimulates target genes such as RANTES, IL-15, and IFNs. Some interferon-stimulated genes (ISGs) are also induced, one of which ISG56 has been linked to inhibition of protein synthesis. Induction of DNA-PK and MAP KKK-related pathways following treatment with stress-inducers leads to N-terminal phosphorylation of IRF-3 to which no functional roles have been assigned yet. Components of bacterial cell wall, such as LPS, activate TLR4 signaling, leading to cytokine production through the activation of JNK/p38 and IKK complex. Dashed lines represent uncharacterized signaling pathways.

downstream of well defined stress-activated kinase cascades comprised of MKK4,7 and MKK3,6 respectively [15–17] (Fig. 1). The heterodimers ATF-2/c-Jun are expressed as nuclear proteins that are activated by phosphorylation of their activation domains by p38/JNK (Fig. 1).

3. Activation of NF-κB by viruses and dsRNA

The NF-κB factors are retained in the cytoplasm of non-stimulated cells in association with inhibitory subunits—inhibitors kappa B (IκBs); dsRNA- and virus-induced phosphorylation at conserved N-terminal residues (Ser 32 and 36) of IκBα is accomplished by the IκB kinase (IKK) complex (Fig. 1). Phosphorylation triggers a signal that induces ubiquitin-dependent, proteasome-mediated degradation of IκBα, and subsequent nuclear translocation of the NF-κB dimers [18]. The rate limiting step in this process is the activation of the IKK complex, which is composed of two catalytic subunits—IKKα and IKKβ and one regulatory subunit IKKγ. Studies indicate that the IKKβ catalytic subunit is required for IKK and subsequent NF-κB activation by dsRNA and viral infection [15,19].

Interestingly, the link between dsRNA and activation of the IKK complex remains a matter of debate. The intracellular target of dsRNA is PKR, a Ser/Thr kinase that is induced by IFNs and activated catalytically through the binding of dsRNA [2]. Following interaction with dsRNA, PKR becomes autophosphorylated and, in turn, phosphorylates a restricted number of substrates, the best characterized of which is the α subunit of eukaryotic protein synthesis initiation factor 2 (eIF2α). Phosphorylation of eIF2α on Ser 51 causes a dramatic inhibition of protein synthesis by sequestering the guanine nucleotide exchange factor eIF2B, a rate-limiting component of the translation machinery (Fig. 1) [2]. Another putative target of PKR is the IKKβ subunit of the IKK complex that physically associates with PKR [20] and is believed to mediate the NF-κB activation in a manner that is independent of PKR kinase activity [15,20]. However, a recent study by Magun's group demonstrated dsRNA-dependent, PKR/RNase L-independent pathways involved in NF-κB activation [21] suggesting the existence of another receptor/effector which recognizes dsRNA. This idea was recently supported by a study demonstrating that the mammalian Toll-like receptor (TLR)-3 recognizes dsRNA. Activation

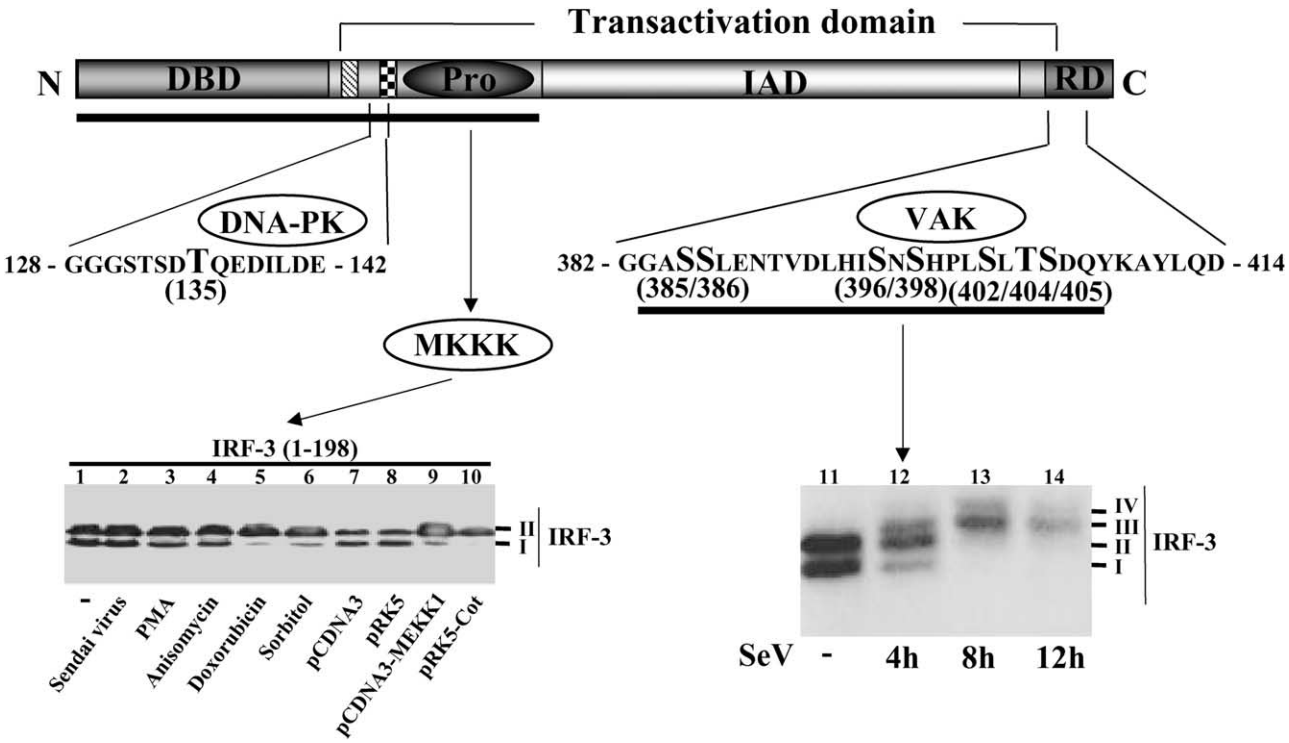

Fig. 2. Schematic representation of IRF-3 structural domains. Different IRF-3 domains are shown: the nuclear localization sequence (oblique bars), the nuclear export sequence (hatched box), the DNA binding domain (DED), the proline rich sequence (Pro), the IRF association domain (IAD), and the signal response domain (RD). Sequences of aa 128–142 and 382–414 are amplified below the schematic. The amino acids targeted for phosphorylation are shown as larger letters. In immunoblot analysis, IRF-3 is expressed as two forms in uninfected cells (form I and II, lane 11), whereas two slower migrating forms of IRF-3 (forms III and IV, lanes 12–14) appear following virus infection corresponding to C-terminal phosphorylation by VAK. IRF-3 C-terminal phosphorylation results in homodimerization, accumulation in the nucleus through CBP association, activation of genes through DNA binding and degradation by the proteasome pathway (see lane 14). DNA-PK phosphorylates Thr 135 near the nuclear export sequence and may inhibit export of IRF-3 from the nucleus. N-terminal phosphorylation of phosphoacceptor site(s) in the region aa 186–198 also occurs in response to stress-inducers, DNA damaging agents and phorbol ester treatment, *via* a MAP KKK-related pathway resulting in the increased appearance of IRF-3 form II in immunoblot analysis (lanes 3–6, 9, 10).

of TLR-3 induces the activation of both MAPK and NF-κB pathways, independently of the adapter protein MyD88 [22] (see below and Fig. 3).

4. IRF-3 activation following virus infection

The signaling pathways leading to IRF-3 phosphorylation and activation remain to be elucidated. IRF-3 is known to be activated in response to virus infection, but recent studies indicated that IRF-3 may also be a phosphorylation target following stimulation of cellular stress pathways or the engagement of TLR receptors (Figs. 1–3).

IRF-3 is a 427 amino acid phosphoprotein that is constitutively expressed in two forms (forms I and II) of about 55 kD when resolved by SDS–PAGE [10,14] (Fig. 2). Upon viral infection, IRF-3 is phosphorylated within the C terminus of the protein on Ser 385, 386 [13] but also on Ser residues 396, 398, 402, 405, and Thr 404 [10], the latter inducing phosphorylated forms III and IV which are detectable on SDS–PAGE [14] (Fig. 2). The C-terminal phosphorylation is thought to produce a change in protein conformation that reveals the IRF association domain and the DNA binding domain, thus promoting dimerization and binding to IRF-3 5′-GAAAC/GC/GGAANT/C-3′ consensus DNA binding site [10,13,23]. In addition, IRF-3 C-terminal phosphorylation is required for association with the histone acetyltransferase nuclear proteins CBP and p300 [10,13,23] causing IRF-3, which normally shuttles into and out of the nucleus, to become predominantly nuclear [9,10,13]. The activated form of IRF-3, bound to CBP, induces transcription through distinct positive

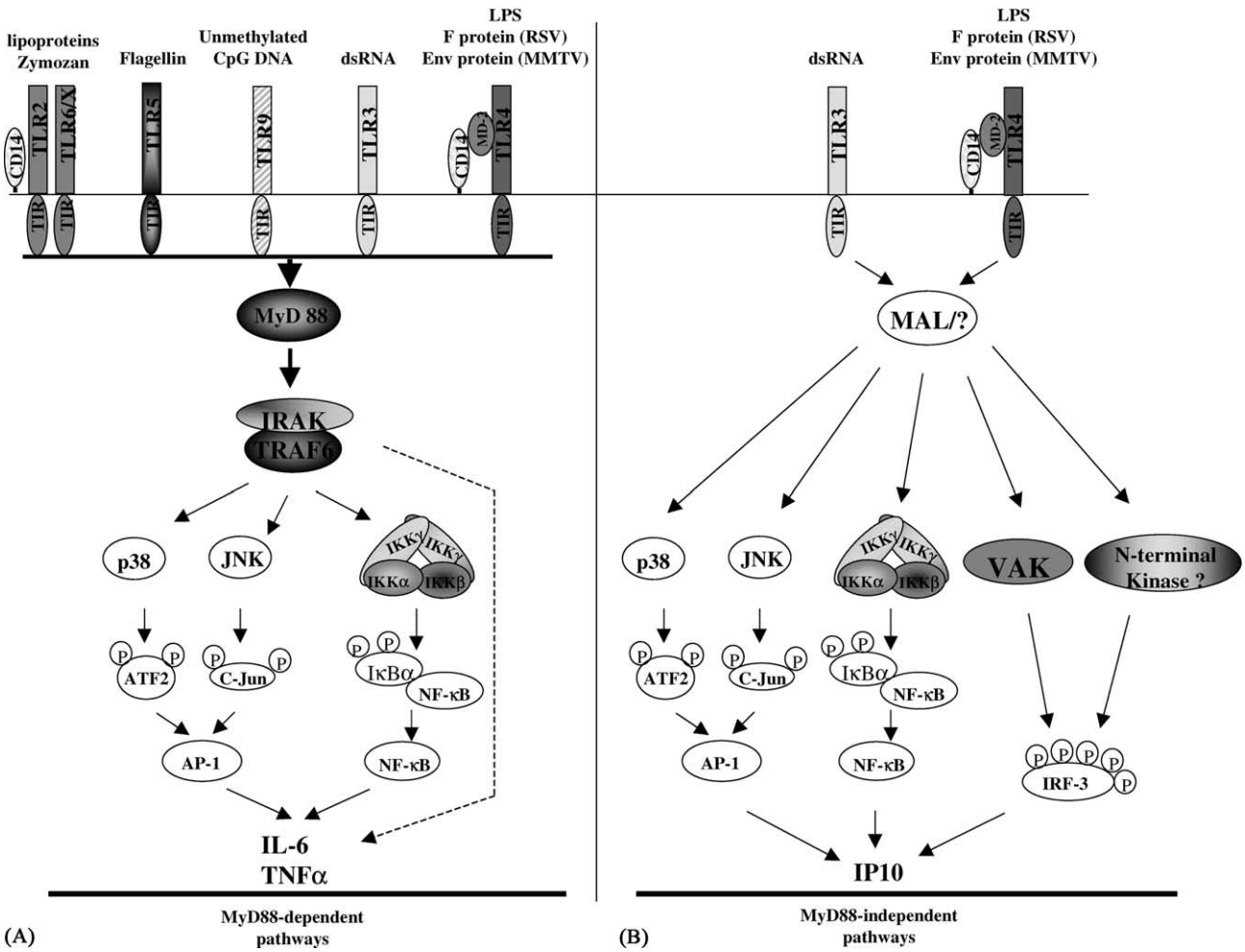

Fig. 3. Signaling through the TLRs. Molecular components involved in TLR signaling are shown (A and B). Activated TLRs associate with a cytoplasmic adapter molecule, MyD88, through the homophilic interaction between their TIR domains. MyD88 interacts with the Ser/Thr kinase IRAK which subsequently activates the TRAF6 adapter. TRAF6 in turn activates the stress-related MAPK pathways JNK and p38, as well as the IKK complex leading to AP-1 and NF-κB activation respectively, and the induction of cytokines such as TNF-α and IL-6 (A). TLR3 and TLR4 also activate MyD88-independent pathways (B). IKK, JNK, and an unidentified IRF-3 kinase can be induced in the absence of MyD88 and TRAF6 and lead to the induction of IFN-inducible genes, possibly through the adapter molecule MAL. The fact that TNF-α and IL-6 production is totally abrogated in the MyD88-independent pathway under conditions where NF-κB and AP-1 are still activated suggests that an unidentified pathway in addition to p38/JNK and IKK is essential for cytokine production (dashed line in A).

regulatory domains in the type 1 IFN promoters, and through select ISRE sites [10,12,13,23–26]. Finally IRF-3 is degraded by a proteasome-mediated mechanism [10,27] (see Fig. 2, lane 14).

Genes targeted by IRF-3 include not only the classical IRF-responsive genes *IFNα1* and *β* but also new candidates such as genes encoding for the chemokine RANTES and the cytokine IL-15 (Fig. 1). These secreted proteins serve to recruit cells of the host immune system to the site of infection and provide a means to eliminate infected cells. Recently, the IFN-stimulated gene *ISG56* was shown to be a direct target of IRF-3 [28]. Interestingly, ISG56 acts as an inhibitor of cell proliferation by downregulating protein synthesis through interaction with the p48 subunit of eIF-3 [29]. IRF-3 5D, a constitutively active form of IRF-3 in which the Ser-Thr cluster (aa 396–405; see Fig. 2) is mutated to the phosphomimetic aspartic acid has previously been shown to induce apoptosis when overexpressed in cell lines [30,31]. Thus, the translation inhibitory function of ISG56 may provide, at least in part, a mechanistic explanation for the ability of IRF-3 5D to mediate apoptosis [29].

The virus-activated kinase (VAK) responsible for IRF-3 phosphorylation has yet to be identified. However, pharmacological and molecular studies suggest that it may be a novel serine/threonine kinase activated in response to virus infection [14,17,32]. VAK represents a component of the cellular machinery that recognizes the viral pathogen and, like the IKK complex, p38 and JNK, activates transcription factors involved in the immediate early response to viral infection (see Fig. 1). Despite the existence of many studies that claim to identify viral and non-viral activators of IRF-3, only Sendai Virus (SeV), Measles Virus (MeV), Newcastle Disease Virus (NDV), Vesicular Stomatitis Virus (VSV), and Respiratory Syncytial Virus (RSV) have been clearly shown to induce forms III and IV through C-terminal phosphorylation [14,33] (Fig. 2). The fact that this list is restricted to closely-related, negative-stranded, enveloped RNA viruses suggests that the C-terminal phosphorylation of IRF-3 may be due to a specific component of the viral life cycle. Indeed, a recent study has shown that the N nucleocapsid (N) protein of MeV induced IRF-3 activation *via* the induction of VAK [34]. Interestingly, N protein physically interacts with IRF-3, implying that IRF-3 itself represents the molecule that detects the viral pathogen *via* nucleocapsid recognition.

A role for dsRNA in IRF-3 activation has also been demonstrated [12,13,26,35]. The dsRNA mimetic poly(I:C) has the capacity to induce a subset of ISGs and cytokines that function to impede viral replication. Indeed, one of the ISRE-binding factors induced by dsRNA is DRAF1, which is composed of IRF-3 and coactivators p300/CBP [26,31]. This observation suggests that dsRNA is sufficient for activation of IRF-3 as well as NF-κB and ATF2/c-Jun (Fig. 1). This idea is strengthened by the fact that overexpression of viral proteins with RNA-binding activities—E3L of vaccinia virus and NS1 of Influenza virus—abrogate IRF-3 phosphorylation and transactivation [32,36]. The antagonist activity of these RNA-binding proteins may be related to their capacity to sequester dsRNA generated during virus replication. However, other proteins with RNA-binding activities such as mammalian Staufen and DRBP76 have no effect on IRF-3 phosphorylation, indicating that the sequestration of RNA may not be the only function associated with E3L and NS1 inhibition [32]. Indeed, PACT, a dsRNA-binding protein originally identified as an activator of PKR, was shown to enhance IRF-3 activation [37]. The possibility that IRF-3 is directly targeted by PKR has been ruled out [14,32]. Studies in PKR null cells, as well as null cells of other PKR-like family members, failed to block virus induced IRF-3 phosphorylation [26,32], thus implying that dsRNA may modulate VAK activity in a PKR-independent manner (Fig. 1).

5. IRF-3 activation following LPS treatment

Identification of Toll as an essential receptor in innate immune recognition in *Drosophila* led to the discovery of a TLR in humans designated TLR4 [38,39]. Subsequent studies have identified a family of 10 members (TLR1–TRL10). Components of the transmembrane TLR signaling pathway are conserved from yeast to mammals and recognize Pathogen Associated Molecular Patterns (PAMPs) found in bacteria and fungi (see Fig. 3).

- TLR4 is essential for the recognition of LPS, the major component of the outer membrane of Gram-negative bacteria [38]. Recent studies also demonstrated that TLR4 functions in the recognition of virus components. The innate immune response to RSV is mediated by the interaction of fusion protein of RSV with TLR4 and CD-14 [40] and activation of B cells by mouse mammary tumor virus is accomplished by interaction between viral envelope protein and TLR4 [41].
- TLR2 in conjunction with TLR6, and possibly other TLRs, is essential for the recognition of lipoproteins and peptidoglycan molecules found on pathogens such as Gram-negative bacteria, Gram-positive bacteria, mycobacteria, spirochetes, and zymozan from fungi [39].
- TLR3 is associated with the binding and activation of dsRNA signaling [22].
- TLR5 is responsible for the recognition of flagellin, a monomeric constituent of bacterial flagella [39].
- TLR9 is essential for the recognition of bacterial CpG DNA, a motif found in non-methylated bacterial DNA [39,51].

A cytoplasmic region of all TLRs is highly homologous to a domain found in the IL-1 receptor, a domain termed the Toll/IL-1 receptor homologous region, TIR (Fig. 3). This structural similarity results in functional similarity since both receptor families use common adaptor/effector pro-

teins such as MyD88, a Ser/Thr IRAK, and TNFR-associated factor (TRAF) 6 to transmit the membrane-generated signal through the cytoplasm to the nucleus (Fig. 3). Indeed, following PAMP interaction with the TLRs, the IKK and JNK/p38 pathways are activated resulting in the activation of NF-κB and AP-1 transcription factors which ultimately lead to cytokine production (Fig. 3A) [1,38]. Treatment of murine macrophages with LPS also induces the production of IFNβ, suggesting the activation of some IRF members by LPS [4,5] and also implying a role for ISGs in response to bacterial infection. In this context, Navarro and David [42] reported that LPS treatment of human U373 astrocytoma cells resulted in IRF-3 activation (nuclear translocation and DNA binding activity) *via* a TLR receptor and p38 dependent pathway. Recently, Akira's group observed induction of the *IP-10* gene with lipid A, the functional moiety of LPS, in MyD88-deficient peritoneal macrophages in which production of TNF-α and IL-6 in response to LPS is completely impaired [43]. In this model however, delayed activation of IKK and JNK and activation of IRF-3 were still observed. These data suggest that a MyD88-independent pathway(s) mediates NF-κB, JNK/p38, and IRF-3 activation in response to TRL4 signaling (see Fig. 3B). Recently, MyD88-adapter-like (MAL) was identified as another essential effector in TLR4 signaling [44]. MAL is thus an attractive candidate as the adapter responsible for the MyD88/TRAF6-independent pathway (Fig. 3B). These observations also indicate that an unknown pathway in addition to NF-κB and MAPK may be required for TNF-α and IL-6 production, a conclusion also reached for the effect of dsRNA, mediated *via* TLR3 [22] (see Fig. 3A). Another target gene, called *UBP43* was also recently shown to be induced by LPS *via* IRF-3 in the murine macrophage-like cell line RAW 264.7 [45]. In apparent contradiction to these observations, Pitha's group reported that LPS inhibits the virus-mediated activation of IRF-3 [46]. It is possible that pretreatment with LPS in this study desensitized the cells to virus infection, particularly if essential TLR signaling components are used by both viruses and LPS.

How LPS activates IRF-3 remains to be determined. Treatment of target cells with LPS activates multiple signaling pathways in addition to IKK/JNK/p38, such as protein kinase C, Src-type tyrosine kinases, and the phosphatidylinositol 3-kinase-protein kinase B pathways [1]. Phosphorylation of IRF-3 in response to LPS was demonstrated by Akira's group [43]. However, the analysis of IRF-3 phosphorylation was not resolved sufficiently to delineate the different IRF-3 phosphorylated forms (see Fig. 2, lanes 11–14). As described previously, virus infection clearly induces C-terminal phosphorylation, whereas stress-inducers (as described below) induce N-terminal IRF-3 phosphorylation to which no convincing function has been assigned. N-terminal phosphorylation might be induced by LPS treatment and therefore involve a kinase other than VAK in IRF-3 activation (Fig. 3B).

6. IRF-3 phosphorylation following stimulation with stress-inducing agents

Stress inducers and DNA damaging agents may also functionally activate IRF-3. Kim *et al.* [47] demonstrated that stress inducers and genotoxic agents such as DNA damaging agents doxorubicin and UV radiation stimulated IRF-3 phosphorylation, nuclear translocation, CBP association, and transcriptional activation of an IRF-3 responsive promoter. In addition, a small molecule CG18 that stimulates MEKK1 activity activated the stress-mediated signaling pathway and stimulated the formation of the IFN-β enhanceosome [48], including the activation of ATF-2/c-Jun, IRF-3, and NF-κB. In these studies, MEKK1 activated IRF-3 through the JNK pathway but not through p38 or IKK pathways. These experiments imply that MEKK1 can induce IRF-3 and ATF2/c-Jun through the JNK pathway and NF-κB through the IKK pathway, resulting in the integration of multiple signal transduction pathways leading to the proper assembly of the IFN-β enhanceosome. However, the phosphorylation site(s) targeted by the MEKK1-related pathway are distinct from the C-terminal sites, since the IRF-3 (5A) protein, in which the five phosphoacceptor sites in the C-terminal end (Ser 396 to Ser 405) were mutated to alanine (see Fig. 2), was still phosphorylated in response to CG18 and MEKK1 [48].

Phosphorylation of IRF-3 by the DNA damaging agent doxorubicin, by the osmotic shock inducer sorbitol, the stress inducer anisomycin, the phorbol ester PMA and overexpression of the MAP KKK family members MEKK1 and Cot has also been observed with phosphorylation occurring at the N-terminal end, between aa 186–198 [14] (Fig. 2). The phosphoacceptor site remains to be identified as does the functional role of this posttranslational modification. Indeed, as opposed to the observation made by Kim's group, stress-inducers and DNA damaging agents failed to induce nuclear accumulation through CBP association or transcriptional activation of RANTES promoter [14].

The unexpected phosphorylation of IRF-3 in the DNA damage response following UV exposure or treatment with doxorubicin might also involve kinases such as the PI3-kinase-like proteins ATM, ATR, and DNA-PK, that are all known to be activated in response to DNA damage. Recently, Karpova *et al.*, identified Thr-135 as a target for phosphorylation by DNA-PK (see Figs. 1 and 2) and also showed that IRF-3 is phosphorylated on Thr-135 following virus infection, resulting in nuclear retention of IRF-3 [49]. This interesting observation raises several important questions: does phosphorylation of Thr 135 by DNA-PK increase binding of IRF-3 to coactivators, or does another mechanism explain IRF-3 nuclear retention; is Thr 135 also phosphorylated by CpG DNA which directly activates DNA-PK and TLR9 (see Fig. 3A) [50,51]; does Thr 135 phosphorylation modulate gene expression?

IRF-3 functions regulated by N-terminal phosphorylation, either through MAP KKK or DNA-PK remain also to

be elucidated. Several scenarios are possible: (1) N-terminal phosphorylation by the stress-induced pathway may alter IRF-3 conformation, thus making the C-terminal Ser-Thr cluster more accessible to VAK; (2) N-terminal phosphorylation may control IRF-3 activity at a step preceding nuclear translocation, such as relief of autoinhibition or dimerization [23]; and (3) N-terminal phosphorylation may regulate stability of the protein.

7. Conclusion

The precise delineation of the signaling pathways that induce IRF-3 activation through C-terminal phosphorylation still await discovery. At this stage, it appears unlikely that a single kinase phosphorylates all the C-terminal phosphoacceptor sites involved in IRF-3 activation (Fig. 2). Thus, multiple signaling pathways activated following virus infection may converge on different IRF-3 sites, possibly in a precise temporal order. The activation of IRF-3 by LPS is intriguing, complicated by the fact that in other studies no induction of IFNβ was observed when embryonic fibroblasts were exposed to LPS [15]. Thus, further experiments are required to clarify the role of TLR4 signaling in IRF-3 activation. Other questions also arise: are other PAMPs such as CpG DNA, flagellin, and zymozan able to activate IRF-3? By which mechanism does dsRNA activates IRF-3? Is N-terminal phosphorylation of IRF-3 by DNA-PK or MAPKKK functional in the context of cytokine induction or does N-terminal phosphorylation function in the regulation of apoptosis? These and other questions are high on the priority list of investigators analyzing this important host response to pathogenic insults.

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