Insights from vaccinia virus into Toll-like receptor signalling proteins and their regulation by ubiquitin: role of IRAK-2

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Abstract
TLRs (Toll-like receptors) are an important class of pathogen-sensing proteins, which signal the presence of a pathogen by activating transcription factors, such as NF-κB (nuclear factor κB). The TLR pathway to NF-κB activation involves multiple phosphorylation and ubiquitination events. Notably, TRAF-6 [TNF (tumour necrosis factor)-receptor-associated factor-6] Lys63 polyubiquitination is a critical step in the formation of signalling complexes, which turn on NF-κB. Here, the relative role of different IRAKs [IL-1 (interleukin 1)-receptor-associated kinases] in NF-κB activation is discussed. Further, I demonstrate how understanding one molecular mechanism whereby vaccinia virus inhibits NF-κB activation has led to a revealing of a key role for IRAK-2 in TRAF-6-mediated NF-κB activation.

Innate immune signalling pathways activated by pathogens
The question of how the immune system initially detects the presence of pathogens has been widely studied over the last 10 years. It is now known that conserved molecular moieties on pathogens, termed PAMPs (pathogen-associated molecular patterns), trigger the activation of host PRRs (pattern-recognition receptors), leading to the initiation of the innate immune response. This response involves early cytokine, chemokine and IFN (interferon) release from sentinel cells expressing PRRs and capable of responding to PAMPs. Families of both membrane-bound and cytosolic PRRs have been identified. These include TLRs (Toll-like receptors), which respond to bacterial, fungal and viral PAMPs, NOD (nucleotide-binding oligomerization domain)-like receptors, which largely respond to intracellular bacteria, and RIG (retinoic acid-inducible gene)-like receptors, which respond to the presence of viral PAMPs in the cytosol [1]. Of these, the TLRs are so far the best characterized in terms of the nature of the PAMPs they respond to and the downstream signalling pathways activated, leading to altered gene expression.

Key words: intracellular signalling, interleukin-1-receptor-associated kinase (IRAK), nuclear factor κB (NF-κB), Toll-like receptor, tumour necrosis-factor-receptor-associated factor-6 (TRAF-6), vaccinia virus.

Abbreviations used: AP-1, adaptor protein-1; CYLD, cylindromatosis; DUB, deubiquitinase; IFN, interferon; IL-1, interleukin-1; IL-1R, IL-1 receptor; IRAK, IL-1 receptor-associated kinase; IFN, IFN regulatory factor; IκB, NF-κB inhibitor; IκBα, IκBα kinase; IL-1, interleukin 1; IL-1R, IL-1 receptor; IRAK, IL-1 receptor-associated kinase; IFN, IFN regulatory factor; IκB, NF-κB inhibitor; IκBα, IκBα kinase; IL-1, interleukin 1; IL-1R, IL-1 receptor; IRAK, IL-1 receptor-associated kinase; IFN, IFN regulatory factor; IκB, NF-κB inhibitor; IκBα, IκBα kinase; IL-1, interleukin 1; IL-1R, IL-1 receptor; IRAK, IL-1 receptor-associated kinase; IFN, IFN regulatory factor; IκB, NF-κB inhibitor; IκBα, IκBα kinase; IL-1, interleukin 1; IL-1R, IL-1 receptor; IRAK, IL-1 receptor-associated kinase; MyD88, myeloid differentiation factor 88; MyD88, myeloid differentiation factor 88; Mal, MyD88 adaptor-like; NF-κB, nuclear factor κB; NEMO, NF-κB essential modulator; PAMP, pathogen-associated molecular pattern; PRR, pattern-recognition receptor; TAK1, transforming growth factor β-activated kinase; TRAM, tumour-necrosis-factor-receptor-associated factor; TRAF, TRAM-interacting protein with a forkhead-associated domain; TRIF, TLR4-TRIF-like receptor; TRAF, TRIF TRIF-related adaptor molecule; VACV, vaccinia virus.

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Regulation of innate signalling to NF-κB by phosphorylation and ubiquitination
The molecular mechanism of IL-1R and TLR signalling to NF-κB has been intensely studied and some aspects of the pathway have been characterized in great detail. This has revealed key roles for both phosphorylation and ubiquitination at multiple points. The IL-1R and TLRs have an intracellular signalling domain termed the TIR (Toll/IL-1R) domain. Upon ligand binding, conformational changes in
the TLR dimer bring the TIR domains into closer proximity, which establishes a new signalling surface that can then recruit, via TIR–TIR interactions, various combinations of TIR domain-containing adaptor proteins. These are MyD88 (myeloid differentiation factor 88) for the IL-1R, TLR7, TLR8 and TLR9; MyD88 and Mal (MyD88 adaptor-like) for TLR2 heterodimers; TRIF (TIR domain-containing adaptor protein inducing IFN-β) for TLR3; and Mal, MyD88, TRIF and TRAM (TRIF-related adaptor molecule) for the TLR4 receptor complex [2].

The formation of these receptor/adaptor complexes then triggers the recruitment of further signalling molecules. For TLR pathways involving MyD88 and Mal, IRAK (IL-1R-associated kinase) family members interact with the adaptors and transduce the signal downstream. Four IRAKs have been identified, being IRAK-1, IRAK-2, IRAK-M and IRAK-4. Both IRAK-1 and IRAK-4 are active serine/threonine kinases, and phosphorylation of IRAK-1 by IRAK-4 is crucial for IRAK-1 activation during TLR signalling, whereas IRAK-2 and IRAK-M are pseudokinases, but latter being a negative regulator of TLR signalling. An important downstream target for IRAK action on the NF-κB pathway is TRAF-6 [TNF (tumour necrosis factor)-receptor-associated factor-6]. How exactly IRAKs participate in the activation of TRAF-6 function is discussed below.

TRAF-6 is thought to be critical for many if not all TLR pathways to NF-κB. It functions upstream of the central NF-κB activating kinase complex, the IKK (IκB kinase) complex, which contains the kinases IKKα and IKKβ, associated with an essential scaffold protein, NEMO (NF-κB essential modulator). IκB sequesters NF-κB dimers in the cytoplasm, and phosphorylation of IκB by the IKK complex targets it for Lys63-linked ubiquitination and subsequent degradation by the proteasome. Thereafter, NF-κB translocates to the nucleus to activate its target genes. The IKK complex also phosphorylates the NF-κB subunit p65, which is necessary for transactivation of genes. The steps between TRAF-6 and downstream IKK activation have recently been characterized in detail, and shown to also critically involve ubiquitination. TRAF-6 is now known to be a RING (really interesting new gene)-domain ubiquitin E3 ligase that functions with an E2 enzyme complex containing Ubc13 and Uev1A to catalyse the formation of Lys63-linked polyubiquitin chains on target proteins, such as NEMO and TRAF-6 itself [3]. Lys63-linked polyubiquitin, in contrast with Lys48-linked ubiquitination, facilitates protein interactions and the activation of signalling cascades [3]. The kinase that activates the IKK complex in the TLR pathways is TAK1 (transforming growth factor-β-activated kinase). TAK1 is recruited to TRAF-6 via two TAK-binding proteins TAB2 and TAB3, which can recognize Lys63 polyubiquitin chains on TRAF-6 [3]. Mutations of the ubiquitin-binding domain of TAB2 or TAB3 abolish their ability to bind polyubiquitin chains on TRAF-6, as well as their ability to activate TAK1 and IKKβ [4]. Recruitment of TAK1 to this complex may allow transphosphorylation of TAK1 via a dimer proximity mechanism [5]. IKK is recruited to the protein complex since NEMO also binds to Lys63 polyubiquitin chains [5]. TAK1 then phosphorylates IKKβ in its activation loop, leading to IKK activation.

Although it has been shown that Lys63-linked TRAF-6 autoubiquitination is essential for downstream IKK activation, recently the role of Ubc13 in this process in vivo has been questioned, since it was found that for macrophages, NF-κB activity was normal in Ubc13-deficient cells, whereas MAPK activation was impaired [6,7]. Further, how exactly TRAF-6 E3 ligase activity is turned on by upstream TLR/adaptor signalling is still unclear, although IRAKs have been proposed to be involved (see below). One likely mechanism for activating TRAF-6 E3 ligase activity is ligand-induced oligomerization of TRAF-6. Evidence for this comes from a study with TIFA (TRAF-interacting protein with a forkhead-associated domain), which is a TRAF-6-interacting protein identified by a yeast two-hybrid screen [8]. Recombinant TIFA was capable of activating IKK in an in vitro reconstitution system containing TRAF-6, TAK1 and other proteins required for ubiquitination [9]. Furthermore, higher-molecular-mass forms of TRAF-6 had higher ubiquitin ligase activity, and TIFA induced the oligomerization and polyubiquitination of TRAF-6 [9]. Thus, in IL-1/TLR pathways, TIFA or proteins of similar function may induce TRAF-6 oligomerization, which may be a prerequisite for its E3 ligase activity.

**Insights into TLR signalling and ubiquitination from VACV (vaccinia virus)**

Prior to the knowledge that TRAF-6 activation involved its E3 ligase activity, IRAK-1 was assumed to be the key upstream activator of TRAF-6. IRAK-4 is the upstream kinase that phosphorylates and activates IRAK-1, and IRAK-4 and IRAK-1 seem to be involved to some degree in signalling by all IL-1R/TLR family members (with the notable exception of TLR3 [10]). Upon receptor ligation, IRAK-4 is recruited to the receptor complex, and then phosphorylates and activates IRAK-1. IRAK-1 is then probably primed to allow interaction with TRAF-6 (either within the receptor complex, or after dissociation from it), and indeed IRAK-1 has TRAF-6-binding motifs that are required for overexpressed IRAK-1 to activate an NF-κB reporter gene [11]. Although assumed, the observation that IRAK1 stimulates TRAF-6 ubiquitin ligase activity has to our knowledge never been reported. In fact, a study in 2006 showed that the Epstein–Barr virus protein LMP1 (latent membrane protein 1), a known viral activator of NF-κB, induced TRAF-6 polyubiquitination and IKKβ activation in IRAK-1-null cells [12].

We have recently proposed that IRAK-2, rather than IRAK1, is the key IRAK that mediates increased TRAF-6 polyubiquitination in IL-1R/TLR signalling. This proposal originated from the approach of studying how VACV proteins participate in evading and subverting the innate immune response, and elucidating the molecular mechanisms of how this occurs. VACV is a large dsDNA virus that expresses multiple immunomodulatory proteins. One such
protein, A52, was found to be an inhibitor of all IL-1R/TLR pathways to NF-κB activation [13–15]. This suggested that its cellular target would be a critical and shared signalling protein for the IL-1R/TLR–NF-κB signalling axis. A52 was found to interact with both IRAK-2 and TRAF-6, but not IRAK-1 [14,16]. Further work showed that the IRAK-2 interaction was sufficient for the ability of A52 to inhibit NF-κB, whereas the TRAF-6 interaction actually facilitated A52-mediated induction of MAPKs [13,16]. Thus A52, which inhibits all IL-1R/TLR pathways to NF-κB, does so via targeting IRAK-2. Further, knockdown of IRAK-2 expression by siRNA (small interfering RNA) suppressed TLR3-, TLR4- and TLR8-induced NF-κB activation in human cell lines, and LPS/TLR4-mediated IL-8 production in primary human cells [13].

Interestingly, expression of IRAK-2, but not IRAK-1, led to TRAF-6 polyubiquitination. Further, IRAK-2 loss-of-function mutants, with mutated TRAF-6-binding motifs, could not activate NF-κB and were incapable of promoting TRAF-6 ubiquitination [13]. Thus we suggest that for the TRAF-6–TAK1–IKKβ signalling axis to NF-κB, IRAK-2 plays a more central role than IRAK-1. Little is known about how IRAK-2 is activated by the upstream TLR complex, and in fact for TLR3, IRAK-2 seems to be pre-associated with the receptor prior to ligand stimulation [13]. Further, the mechanism whereby IRAK-2 would activate or increase TRAF-6 E3 ligase activity remains to be determined, but as with TIFA (see above), this may involve mediating TRAF-6 oligomerization.

Redefining the role of IRAK-1 versus IRAK-2

So, what of the role of IRAK-1 in NF-κB activation? Clearly, IRAK-1 is still likely to be involved to some degree, but this may be in a more subtle and pathway-specific manner than was previously envisaged. For example, in the LMP1 study noted above, although LMP1-stimulated TRAF-6 polyubiquitination and IKKβ activation were IRAK-1–independent, IRAK-1 was required for LMP1-induced p65 phosphorylation (a necessary step in rendering NF-κB capable of transactivating genes) [12]. IRAK-1 has also been shown to be itself Lys63-polyubiquitinated (probably by Pellino proteins, which have E3 ligase activity [17,18]), which probably allows IRAK-1 to engage with the IKK complex via a direct interaction with NEMO [18]. These roles for IRAK-1 may only operate in some but not all TLR pathways since for TLR9 in dendritic cells, IRAK-1 was dispensable for NF-κB activation and pro-inflammatory cytokine production [19], while another study showed that the absence of IRAK-1 had no effect on LPS induction of the NF-κB-dependent pro-inflammatory cytokine, IL-1β [20].

Further, alternative NF-κB-independent functions for IRAK-1 have emerged, such as TLR4-mediated STAT3 (signal transducer and activator of transcription 3) activation and up-regulation of IL-10 [20], and TLR-7, -8 and -9-mediated IRF-7 activation [7]. In contrast, the lack of any effect of A52 on TLR-mediated IRF activation suggests that IRAK-2 has no role in IRF activation. Thus, in light of the current knowledge of IRAK function, we propose that IRAK-2 is more fundamentally important for NF-κB activation than IRAK-1, while IRAK-1 and not IRAK-2 has a key role in the TLR–IRF axis. IRAK-4, in contrast, may control both the IRF and NF-κB pathways for some TLRs [7].

Conclusions

The role of ubiquitination in signalling is now appreciated, and is likely to be at least as important as phosphorylation. Further, just as both kinases and phosphatases are known to be critical in regulating phosphorylation in signalling pathways, both ubiquitinases and DUBs (deubiquitinases) regulate ubiquitination of signalling proteins. For example, two DUBs known to be important in regulating NF-κB activation are A20 and CYLD (cylindromatosis), and dysregulation of CYLD is linked to tumour development [21].

TRAF-6 Lys63 polyubiquitination is a critical signalling event in the IL-1R/TLR signalling axis to NF-κB, and determining the mechanism whereby the VACV protein A52 inhibits NF-κB ultimately led to the demonstration of the role of IRAK-2 (and not IRAK-1) in mediating this event. Thus a study of the immune evasion strategies of viruses at the molecular level can be informative as to how the host immune signalling pathways operate.

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References


