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The Poxvirus Protein A52R Targets Toll-like Receptor Signaling Complexes to Suppress Host Defense

Mary T. Harte,1 Ismar R. Haga,3 Geraldine Maloney,2 Pearl Gray,1 Patrick C. Reading,3 Nathan W. Bartlett,3 Geoffrey L. Smith,3 Andrew Bowie,2 and Luke A.J. O’Neill1

1The Cytokine Research Group and 2The Viral Immune Evasion Group, Department of Biochemistry, Trinity College, Dublin 2, Ireland
3Department of Virology, The Wright-Fleming Institute, Faculty of Medicine, Imperial College of Science, Technology & Medicine, St. Mary’s Campus, London W2 1PG, United Kingdom

Abstract

Toll-like receptors (TLRs) are crucial in the innate immune response to pathogens, in that they recognize and respond to pathogen-associated molecular patterns, which leads to activation of intracellular signaling pathways and altered gene expression. Vaccinia virus (VV), the poxvirus used to vaccinate against smallpox, encodes proteins that antagonize important components of host antiviral defense. Here we show that the VV protein A52R blocks the activation of the transcription factor nuclear factor-κB (NF-κB) by multiple TLRs, including TLR3, a recently identified receptor for viral RNA. A52R associates with both interleukin 1 receptor–associated kinase 2 (IRAK2) and tumor necrosis factor receptor–associated factor 6 (TRAF6), two key proteins important in TLR signal transduction. Further, A52R could disrupt signaling complexes containing these proteins. A virus deletion mutant lacking the A52R gene was attenuated compared with wild-type and revertant controls in a murine intranasal model of infection. This study reveals a novel mechanism used by VV to suppress the host immunity. We demonstrate viral disabling of TLRs, providing further evidence for an important role for this family of receptors in the antiviral response.

Key words: nuclear factor-κB • signal transduction • vaccinia virus • immunomodulation • Toll-like receptor

Introduction

Members of the IL-1 receptor (IL-1R)/Toll-like receptor (TLR)* superfamily are key mediators in innate and adaptive immunity (1). The superfamily is defined by the presence of a cytosolic motif termed the Toll/IL-1 receptor (TIR) domain (1). The family includes receptors for the proinflammatory cytokines IL-1 and IL-18 as well as the TLR members, which participate in the recognition of pathogens by responding to pathogen–associated molecular

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Abbreviations used in this paper: dsRNA, double-stranded RNA; IP, immunoprecipitation; IRAK, IL-1 receptor–associated kinase; Mal, MyD88 adaptor-like; NF, nuclear factor; TAB, TAK1 binding protein; TAK1, TGF-β–activated kinase 1; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; TRAF, TNF receptor associated factor; VV, vaccinia virus.
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Stimulation of IL-1R/TLR family members with the appropriate ligands leads to activation of NF-κB and also the mitogen-activated protein kinases p38, JNK, and p42/44. The activation of NF-κB by IL-1, IL-18, TLR2, TLR7, and TLR9 is absolutely dependent on the cytoplasmic TIR domain-containing protein MyD88 (16, 18–20), which is recruited to receptor TIR domains (21–23). However, both TLR4 and TLR3 are able to activate NF-κB, by both a MyD88-dependent and MyD88-independent pathway (15, 24). For TLR4, the MyD88-independent pathway may be mediated by MyD88 adaptor-like (Mal, also known as TIRAP), as it has been demonstrated that Mal is recruited to TLR4, and is involved in NF-κB activation (25, 26). Activation of NF-κB by the MyD88-dependent pathway can proceed via recruitment of IL-1 receptor–associated kinase (IRAK) or IRAK2, while Mal can recruit IRAK2 (25). IRAK or IRAK2 activation in turn leads to recruitment of TNF receptor–associated factor 6 (TRAF6). TRAF6 is required for the ubiquitination and activation of TGF-β (TAK1), which, in complex with TAK1 binding protein 1 (TAB1), phosphorylates IκB kinase (IKK) leading to NF-κB activation (27).

Vaccinia virus (VV), the vaccine used to eradicate smallpox, encodes many immunomodulatory proteins to evade the host immune response (28). Previously we demonstrated that VV protein A52R down-regulated the activation of NF-κB by the IL-1R/TLR superfamily members IL-1R, IL-18R, and TLR4 (29). Here we show that A52R inhibits multiple TLR pathways to NF-κB, including TLR3, and provide evidence to suggest the mechanism of action. Further we show that A52R contributes to VV virulence in vivo. Thus, we demonstrate viral targeting of TLR signaling molecules, and reveal a novel mechanism used by VV to suppress the host immune response.

Materials and Methods

Expression Plasmids. Chimeric TLR receptors CD4-TLR1, CD4-TLR2, CD4-TLR4, CD4-TLR5, and CD4-TLR6 composed of the extracellular domain of CD4 fused to the transmembrane domain and cytosolic tail of the TLR were a gift from R. Medzhitov (Yale University, New Haven, CT). TLR3 was a provided by K. Fitzgerald and D. Golenbock (University of Massachusetts Medical School, Worcester, MA). AU1-Myc-MyD88, Myc-IRAK2, and Myc-kIRAK2 expression vectors were a gift from M. Muzio, Mario Negri Institute, Milano, Italy (23). IRAK, Flag-TRAF6, Flag-TRAF6 domain (amino acids 289–522), and Flag-TRAF2 expression plasmids were provided by Tulank Inc. (San Francisco, CA). Flag-TAK-1 and HA-TAB-1 expression plasmids were a gift from H. Sakurai (Tanabe Seiyaku Co., Osaka, Japan). Construction of A52R, Flag-A52R, and HA-Mal expression plasmids was described previously (25, 29).

Antibodies. Polyclonal antibodies were raised against a purified, bacterially expressed glutathione S-transferase (GST) fusion of A52R, encoded by a plasmid synthesized by inserting full-length A52R downstream of GST in the bacterial expression vector GEX4T2. Other antibodies used were Anti-Flag M2 monoclonal antibody, anti-Flag M2 conjugated agarose, anti-myc monoclonal antibody clone 9E10 (all from Sigma-Aldrich), anti-AU1 monoclonal antibody (BabCO), anti-HA polyclonal antibody (Y-11), and anti-TRAF6 (H-274; both from Santa Cruz Biotechnology, Inc.). Anti-IRAK antibody was a gift from K. Ray (GlaxoSmithKline).

NF-κB Reporter Gene Assay. HEK 293 cells (2 × 10⁴ cells per well) were seeded into 96-well plates and transfected the next day with expression vectors, κB-luciferase reporter gene, and Renilla-luciferase internal control as described previously (25). The total amount of DNA per transfection was kept constant at 220 ng by addition of pcDNA3.1 (Stratagene). After 24 h, reporter gene activity was measured (25). Data are expressed as mean fold induction ± SD relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate.

Immunoprecipitation and Immunoblotting. HEK 293T cells were seeded into 100-mm dishes (1.5 × 10⁶) 24 h before transfection.
6-wk-old Balb/c mice were anaesthetized and inoculated with 10^4 PFU of VV in 20 µl of phosphate-buffered saline. A control group was mock infected with PBS. Each day the weights of the animals and signs of illness were measured as described previously (31).

Results

**A52R Inhibits NF-κB Activation by Multiple TLRs.** We have previously shown that A52R blocks the activation of NF-κB by IL-1, IL-18, and TLR4, with little inhibitory effect on the activation of NF-κB by TNF (29). Given the emerging role of TLRs in antiviral responses, we tested the effect of A52R on NF-κB activation by other TLRs. Chimeric versions of the TLRs, comprising the murine CD4 extracellular domain fused to the cytoplasmic domain of a human TLR render TLRs constitutively active, and have proved useful in probing TLR signaling pathways (5, 11, 32). Some TLR cytoplasmic domains can induce gene expression as homodimers (TLR4 and TLR5), while others signal as heterodimers (TLR1, TLR2, and TLR6; references 5 and 11). Using these chimeras TLR signaling can be examined in the absence of exogenous activator. Induction of an NF-κB-dependent reporter gene by overexpression of either CD4-TLR4 or CD4-TLR5, or a combination of either CD4-TLR2 and 6, or CD4-TLR2 and 1 in HEK293 cells was inhibited in all cases by coexpression of A52R (Fig. 1 A).

DsRNA is a molecular pattern associated with viral infection, and TLR3 has been shown to sensitize cells to activation by polyinosine-polycytidylic acid (poly(I:C)), a synthetic dsRNA analogue (15). Therefore, we also tested the effect of A52R on TLR3-dependent NF-κB activation induced by poly(I:C). Transfection of HEK293 cells with TLR3 led to strong dose-dependent activation of NF-κB by poly(I:C), which was not seen in the absence of TLR3 (Fig. 1 B). This
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TLR3-dependent induction of NF-κB was completely blocked by A52R (Fig. 1 B) in a dose-dependent manner (Fig. 1 C). Thus, A52R is a global inhibitor of signaling by the TLR family, with TLR3 being particularly sensitive.

A52R Interacts with TRAF6 and IRAK2. The activation of NF-κB by different TLRs is mediated by a common set of signaling molecules (1). The ability of A52R to inhibit NF-κB activation by multiple TLRs suggested that its effects might be due to its interaction with a molecule whose function is critical to signaling by all TLRs. Thus, the ability of A52R to associate with characterized mediators of signaling of the TLR family was examined in coimmunoprecipitation (coIP) experiments. No complex formation was detected when A52R was coexpressed with MyD88 (Fig. 2 A), Mal (Fig. 2 B), or IRAK (Fig. 2 C), as indicated by the lack of a band in lane 6 in each case. Under the same conditions complexes were detected between MyD88 and IRAK2, Mal and IRAK2, and IRAK and TRAF6 (unpublished data) as has been demonstrated previously (23, 25, 33). However, upon expression of A52R with IRAK2 a clear complex of A52R with IRAK2 was immunoprecipitated, using antibody to either A52R (Fig. 2 D, lane 6), or IRAK2 (unpublished data). The next signaling mediator downstream of the IRAK family is TRAF6 (33). Coexpression of A52R with TRAF6 resulted in the formation of a complex with high stoichiometry, as detected by IP with an antibody to either A52R (Fig. 2 E, lane 6), or TRAF6 (unpublished data). TRAF6 is responsible for activating TAK1 that forms a complex with its two coactivators TAB1 and TAB2 (27). A52R was coexpressed with either TAK1 or TAB1 to determine if it associates with either of these downstream mediators of TRAF6 signaling. A weak but reproducible interaction was detected between A52R and TAK1 (Fig. 2 F, lane 6). No interaction was detectable between A52R and TAB1 (unpublished data). These results indicate that A52R is capable of interacting with both IRAK2 and TRAF6. The interaction with TAK1 is likely to occur via endogenous TRAF6.

A52R Associates with the TRAF6 TRAF Domain. We next examined the specificity and functional consequences of the interaction of A52R with TRAF6-containing complexes. Fig. 3 A shows that A52R could be immunoprecipitated with endogenous TRAF6 (Fig. 3 A, top panel, compare lanes 3 and 4). To determine the regions in TRAF6 responsible for interacting with A52R, truncated versions of TRAF6 were coexpressed with A52R and tested for their ability to interact by IP. A truncated version of TRAF6 composed of just the TRAF domain was able to interact with A52R to the same extent as the full length TRAF6 (Fig. 3 B, lanes 5 and 6, compare top and middle panels). TRAF2 is a TRAF family member with a role in TNF signaling (34). A52R has only minimal inhibitory effect on the activation of NF-κB by TNFR (29) and consistent with this, no interaction between A52R and TRAF2 was detected (Fig. 3 B, bottom panel). A role for TRAF6 in the TLR pathways inhibited by A52R was confirmed by testing the effect of...
A52R inhibits the activation of NF-κB by Mal. 293 cells were transfected with 10 ng Mal where indicated (+), A52R, and NF-κB reporter plasmid. Luciferase activity was measured after 24 h. (D) A52R causes the dissociation of a Mal-IRAK2-containing complex. A52R was cotransfected with IRAK2 and Mal. The amount of IRAK2-Mal complex formed was assessed by immunoprecipitation using anti-HA antibody, followed by immunoblotting with anti-myc antibody. A52R can be seen to interfere with the Mal/IRAK2 complex.

A52R Disrupts IRAK2-dependent Signaling. To test whether the ability of A52R to target TRAF6 could explain inhibition of TLR-induced NF-κB activation, the effect of A52R on the ability of TRAF6 to form active signaling complexes necessary for the activation of NF-κB was assessed. As an example of a TRAF6 complex important for NF-κB activation, we looked at the effect of A52R on a complex containing TRAF6 and TAB1 (27). We detected a TRAF6-TAB1-containing complex by IP (Fig. 3 D, top panel), and as the expression of A52R increased, the amount of TAB1 capable of coIP with TRAF6 decreased steadily (Fig. 3 D, top panel). This effect was specific to TRAF6 because the expression of increasing levels of A52R had no effect on the formation of a TAB1-TAK1 complex (Fig. 3 D, bottom panel).

A52R Disrupts IRAK2-dependent Signaling. Fig. 2 showed that A52R could also interact with IRAK2-containing complexes. We therefore examined the specificity and functional consequences of this interaction. The death domain of IRAK2 was required for association with A52R, as a truncated version of IRAK2 termed kIRAK2, which lacks the death domain, was unable to interact with A52R (Fig. 4 A, top panel, compare lanes 3 and 4). Similar to TRAF6, there was a correlation between inhibition of TLR-induced NF-κB activation by A52R, and a role for IRAK2 in these pathways: Fig. 4 B shows that each CD4-TLR-induced signal that was sensitive to A52R was also blocked by dominant negative IRAK2 (top panel). Further, we obtained the first evidence that IRAK2 has a role in TLR3-dependent poly(I:C)-induced NF-κB activation, as dominant negative IRAK2 dose dependently inhibited this signal (Fig. 4 B, bottom panel). Thus, IRAK2 has a wide-ranging role in many TLR pathways to NF-κB activation, providing a further rationale for the inhibitory effect of A52R on TLR signaling.

The activation of NF-κB by Mal requires IRAK2 (25). Given that IRAK2 is a target for A52R, the effect of A52R on the ability of Mal to activate NF-κB was examined. Overexpression of Mal activated NF-κB, and this activation was inhibited by the coexpression of A52R in a dose-dependent manner (Fig. 4 C). As Mal interacts with IRAK2 (25), we hypothesized that A52R may inhibit the activation of NF-κB by Mal by preventing the formation of an active Mal-IRAK2 signaling complex. Therefore, the effect of A52R expression on the ability of Mal to interact with IRAK2 was tested. As the expression of A52R increased, the amount of IRAK2 capable of coimmunoprecipitation with Mal decreased steadily (Fig. 4 D). This decrease in complex formation was not due to a decrease in the expression of either IRAK2 or Mal since direct immunoblot showed equal expression of both signaling molecules as the expression of A52R increased (unpublished data). Thus, increasing expression of A52R is able to inhibit the acti-
Discussion

In this report we identify the VV protein A52R, as a broad intracellular inhibitor of signaling by TLRs and show it contributes to virus virulence in vivo. In addition to inhibition of the IL-1R, the IL-18R, and TLR4 (29), A52R was shown here to inhibit NF-κB induction by multiple TLRs, including TLR3, which has been implicated in antiviral innate immunity.

There is intense interest in the IL-1R/TLR family, given its emerging central importance in the innate immune response to diverse pathogens (1). During the course of viral infection the body mounts several lines of host defense involving constituents of the IL-1R/TLR superfamily. The cytokines IL-1 and IL-18 are key regulators of the innate and adaptive immune response to viral infection. In particular IL-1 is responsible for inducing a fever response during viral infection, which is antagonized by the production of a soluble IL-1β binding protein (B15R) by VV (31). IL-18 is a potent inducer of IFN-γ, and administration of IL-18 has been shown to elicit antiviral effects in VV-infected mice (35), while deletion of the VV IL-18 binding protein caused virus attenuation in vivo (36). As for the TLR arm of the family, recent work has suggested that they are crucial mediators of an innate immune response to viral infection. TLR3 has been shown to activate NF-κB and the production of IFN-α and IFN-β in response to poly(I:C), a synthetic analogue of dsRNA (15), which is produced by most viruses including VV at some stage in their life cycle. In addition to its role as the receptor that responds to LPS, TLR4 has been shown to mediate the cytokine-stimulating ability of the fusion protein of respiratory syncytial virus (RSV), while RSV replicated to a higher titre and persisted longer in TLR4-deficient mice than in control mice (13). TLR4 is also required by murine retroviruses for B cell activation (14). TLR2 signaling has recently been shown to be activated by the hemagglutinin protein from measles virus (17), while activation of TLR7 by certain imidazoquinoline compounds has been shown to account for the antiviral effects of these compounds (16). It is possible that other TLRs will also have a role in responding to viral infection. If the TLR family is truly important in antiviral host defense, one would assume that viral mechanisms to antagonize this family exist. Therefore, the discovery of VV A52R as an intracellular global inhibitor of TLR signaling lends strong support to the emerging role of TLRs in the host response to viral infection.

A52R could be communoprecipitated with either IRAK2 or TRAF6. The strength of the interaction with each partner suggest that each interaction is direct, rather than A52R interacting with one protein via a ‘bridge’ with the other. This was theoretically possible since IRAK2 and TRAF6 interact (23). Importantly, the interaction of A52R with IRAK2 was able to disrupt IRAK2/Mal interactions, while the interaction with TRAF6 disrupted TRAF6/TAK1 interactions. An important control was a lack of effect on TAB1/TAK1 complexes, showing that the effect of A52R on TRAF6/TAB1 was via TRAF6 and not TAB1.
The work here does not exclude the possibility that A52R might disrupt other important TRAF6- and IRAK2-containing signaling complexes, and thus increase the range of downstream signals blocked by vaccinia. Disruption of signaling complexes by A52R provides a mechanistic explanation for its ability to inhibit TIR-dependent signaling. Some viral proteins, such as the hepatitis C virus nonstructural 5A protein (37) and the rotavirus capsid protein VP4 (38) are known to affect intracellular signaling by targeting TRAF2. However, this is the first demonstration of a viral protein targeting TRAF6. The observation that the TRAF domain of TRAF6 is targeted is of interest. The TRAF domain is responsible for mediating the oligomerization of TRAF6, which is required for its ubiquitination and activation of IKK via TAK (27, 39). A dominant negative version of TRAF6 composed of only the TRAF domain is thought to function by inhibiting oligomerization (33), and it is possible that the binding of A52R functions in a similar manner.

A52R is also the first viral protein identified to target IRAK2. This, together with our demonstration of a role for IRAK2 in all the TLR pathways tested, including TLR3, suggests an important role for IRAK2 in antiviral immunity and TLRs in general. The death domain of IRAK2 was required for the association. Functionally there is a clear difference between the death domain of IRAK and IRAK2. Based on results here, IRAK2 might be predicted to be more important for TLRs involved in the antiviral response than IRAK. Determination of the exact role of IRAK2 in antiviral immunity and TLR signaling awaits the generation of an IRAK2 knockout mouse.

The apparent redundant targeting of two signaling molecules present on common pathways may indicate the critical importance to the virus of inhibiting NF-κB activated by TLRs. However, from recent work using knockout mouse studies it has become clear that although the IL-1R/TLR family share a common pool of downstream signaling molecules, specific molecules are used in different contexts leading to the range of different signals that are generated by TLRs. In response to LPS, TLR4 activates cytokine release from dendritic cells by a MyD88-dependent pathway, whereas NF-κB activation and expression of costimulatory molecules can occur in the absence of MyD88 (40). We recently identified Mal, a novel TIR-containing adaptor protein, which interacts with IRAK2 and can activate NF-κB in response to TLR4 stimulation (25). Consistent with the targeting of IRAK2 by A52R, signaling triggered by Mal was sensitive to inhibition by A52R. Significantly, the MyD88-independent pathway, which may involve this Mal-IRAK2 axis (41), leads to the activation of the antiviral transcription factor IFN-regulatory factor 3 (IRF3) and induction of IRF3-responsive genes, and has been shown to be independent of TRAF6 (42). Thus, there would appear to be at least two types of signaling pathways activated by the IL-1R/TLR superfamily, those dependent on MyD88, IRAK/IRAK2, and TRAF6, and those activated by a Mal/IRAK2-dependent and TRAF6-independent pathway. Thus, the ability of A52R to target both IRAK2 and TRAF6 would significantly increase the range of TIR domain-activated signaling pathways that VV is able to inhibit.

The importance of A52R, and by extrapolation TLRs, to virus virulence was demonstrated by deletion of A52R from VV, which reduced virulence. The attenuation seen by deletion of A52R is similar to that resulting from loss of IL-18 binding protein (36) or the type I IFN binding protein (43).

In conclusion, we have identified the VV protein A52R as an inhibitor of TLR signaling, which acts by a novel mechanism of targeting the two key signaling molecules IRAK2 and TRAF6. The targeting of these two molecules involved in a diverse range of TIR domain-dependent signaling pathways is likely to confer VV with a significant selective advantage in the presence of the host immune system. This study further highlights the emerging importance of TLRs in antiviral innate immunity.

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