Poxviral Protein A46 Antagonises Toll-like Receptor 4 Signaling by Targeting BB Loop Motifs in Toll-IL-1 Receptor Adaptor Proteins to Disrupt Receptor:Adaptor Interactions

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Running title: Mechanism of inhibition of TLR4 by poxviral A46

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Background: TLR4 signaling is inhibited by poxviral protein A46, but the mechanism is unknown.

Results: We identify the protein interaction surfaces within the TLR4 complex that A46 antagonises, and characterize the interaction between A46 and TRAM.

Conclusion: A46 prevents receptor:adaptor interactions, and has a TRAM-specific interaction motif.

Significance: This work reveals the molecular basis for poxviral antagonism of TLR4.

Toll like receptors (TLRs) have an anti-viral role in that they detect viruses, leading to cytokine and IFN induction, and as such are targeted by viruses for immune evasion. TLR4, although best known for its role in recognising bacterial LPS, is also strongly implicated in the immune response to viruses. We previously showed that the poxviral protein A46 inhibits TLR4 signalling and interacts with Toll–IL-1 receptor (TIR) domain-containing proteins of the receptor complex. However the exact molecular mechanism whereby A46 disrupts TLR4 signalling remains to be established, and may yield insight into how the TLR4 complex functions, since viruses often optimally target key residues and motifs on host proteins for maximal efficiency. Here we show that A46 targets the BB loop motif of TIR proteins and thereby disrupts receptor:adaptor (TLR4:Mal and TLR4:TRAM), but not receptor:receptor (TLR4:TLR4) nor adaptor:adaptor (Mal:MyD88, TRAM:TRIF and Mal:Mal) TIR interactions. The requirement for an intact BB loop for TIR adaptor interactions correlated with the protein:protein interfaces antagonised by A46. We previously discovered a peptide fragment derived from A46 termed VIPER (Viral Inhibitory Peptide of TLR4), which specifically inhibits TLR4 responses. Here we demonstrate that the region of A46 from which VIPER is derived represents the TLR4-specific inhibitory motif of the intact protein, and is essential for A46:TRAM interactions. This study provides the molecular basis for pathogen subversion of TLR4 signalling and clarifies the importance of TIR motif BB loops, which have been selected for viral antagonism, in the formation of the TLR4 complex.

The innate immune response to viral pathogens is critical in order to mobilize protective immunity. Cells of the innate immune system detect viral infection largely through recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), the retinoic acid-inducible gene I-like receptors (RLRs), the nucleotide-binding oligomerization domain-like receptors (NLRs) and Absent in melanoma 2-like receptors (ALRs) (1,2). Of the PRRs, the TLRs are the best studied (3). The anti-viral roles of TLR3, -7, -8 and -9, which recognize viral nucleic acids and mount a potent antiviral response by inducing type I IFNs, are well established (4). TLR2, known to recognize bacterial ligands, is also involved in the recognition of viral glycoproteins, leading to the induction of proinflammatory responses (5) and TLR2 has recently been shown to be required in vivo in specific cell types for the induction of type I IFN in response to the poxvirus vaccinia virus (VACV) (6). TLR4, although best known for its role in recognising LPS, also plays a role in the immune response to viruses. For example, vesicular stomatitis virus (VSV) glycoprotein G induces type I IFN in a TLR4-dependent manner (7), while the induction of proinflammatory cytokines by the F protein of respiratory syncytial virus (RSV) is also TLR4-
dependent (8). In terms of poxviruses, TLR4 has been shown to be protective in pulmonary VACV infection (9). In that study, mice lacking TLR4 signaling displayed greater viral replication, hypothermia and mortality than control animals (9). Further, a number of viral proteins that interfere with TLR signaling have been identified, thus highlighting the importance of TLRs in anti-viral immunity (10).

TLRs are type I transmembrane proteins which consist of an ectodomain containing leucine-rich repeats that mediate the recognition of PAMPs, a single-pass transmembrane domain, and an intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domain which is required for downstream signal transduction (11). PAMP binding to the ectodomain induces receptor oligomerization, bringing the juxtamembrane sequences into close proximity and leading to the dimerization of the cytosolic TIR domains (12,13). This activated conformation of the receptor provides a scaffold for the recruitment of TIR domain-containing adaptor proteins that engage downstream signal transduction pathways leading to activation of transcription factors such as NF-κB and IFN regulatory factors (IRFs). TLRs utilise five different TIR domain-containing adaptor proteins for signaling, namely myeloid differentiation primary response gene 88 (MyD88), MyD88-adaptor-like (Mal), TIR domain-containing adaptor-inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α and HEAT-armadillo motif protein (SARM) (14). MyD88 is used by all TLRs except TLR3 (15). The other adaptor proteins fulfill more specialized roles. The most complex adaptor use is displayed by TLR4. Although the exact molecular details remain to be clarified, activation of TLR4 by lipopolysaccharide (LPS) leads to initial recruitment of TRAM and/or Mal. These proteins are thought to function as “bridging” adaptors whereby Mal engages MyD88 (16) and TRAM engages TRIF (17), thus transducing TLR4 signals from the cell membrane or from the endosomal compartment, respectively (18). Crystal structures for the TIR domains from TLR2 (19), TLR10 (20), interleukin-1 receptor accessory protein-like (IL-1RAPL) (21), and Mal (22)(23), as well as the solution NMR structure of the TIR domain from MyD88 (24), have been determined. These studies have identified a number of conserved regions, notably the BB loop that is positioned between the βB strand and the αB helix, which has been shown in some TIR proteins to be essential for functional TLR signaling (25) (26) (16) (27) (28). For TLR4, a P712H mutation in the BB loop is responsible for the unresponsiveness of C3H/HeJ mice to LPS (29). A rationale for this has been provided by TLR4 mutagenesis and modeling studies, which strongly suggests that the TLR4 BB loop is required for TIR-TIR interactions in TLR4 dimer formation (25) (30) (31). Although the BB loop of the TIR adaptors is also likely essential for their TLR signal transducing functions, the exact role of this motif in the adaptors, compared to TLR4, is less clear.

The importance of TLRs in antiviral immunity is emphasized by the fact that viruses target TLRs for immune evasion (10). VACV encodes immunomodulatory proteins which act intracellularly to target various components of innate immune signal transduction pathways. These viral proteins include A46 (32), A52 (33), N1 (34), B14 (35), K7 (36) and C6 (37). Of these, A46 and A52 specifically disrupt TLR signaling, and we previously demonstrated a role for A46 in VACV virulence (32). A46 targets TLR4 in particular, and inhibits LPS-induced MAPK, NFκB and IRF activation. A46 can interact with all the known components of the TLR4 complex that contain a TIR domain, i.e. TLR4 itself and the TIR adaptor molecules Mal, MyD88, TRAM and TRIF (32). A46 is predicted to form a Bcl-2-like fold, since the most significant structural homology was found with the VACV Bcl-2-like proteins A52 and B14 (38) (39). Remarkably, VIPER, which is an 11 amino acid peptide derived from a surface patch of A46 predicted by the Bcl-2 model fused to a cell-penetrating delivery sequence, potently inhibited TLR4 responses, and associated with Mal and TRAM (38). However, the mechanism whereby A46 disrupts normal TLR4 complex function is still unclear.

Here we show that A46 impairs TLR4 signalling by disrupting receptor:adaptor (TLR4:Mal and TLR4:TRAM), but not receptor:receptor (TLR4:TLR4) nor adaptor:adaptor (Mal:MyD88, TRAM:TRIF and Mal:Mal) TIR interactions. Further, we demonstrate that A46 targets the conserved BB loop of TIR proteins and cannot interact with those TIR proteins that do not have this proline. Consistent with this, TLR4 complex receptor:adaptor interactions, but not adapter:adapter interactions, required that Mal and TRAM have an intact proline in their AB and BB loops respectively, and also that TRAM...
have an intact BB loop cysteine. Previous modeling studies have suggested that the region of A46 from which the TLR4-specific inhibitory peptide, VIPER, is derived may be a TLR4-antagonistic site on A46. When this region was disrupted, the anti-TLR4 ability of A46 was lost, as was its ability to interact with TRAM. Thus we identify motifs within TRAM and Mal targeted by poxviruses, demonstrate that these motifs are essential for adaptor engagement with TLR4, and reveal a TLR4-specific inhibitory surface on A46 for TRAM antagonism.

EXPERIMENTAL PROCEDURES

Cell culture – HEK293T cells were purchased from European Collection of Animal Cell Cultures (Salisbury, UK). HEK293 cells stably transfected with IL-1R (HEK293-R1) were a gift from Tularik (San Francisco, CA). HEK293 cells stably transfected with TLR4, MD2 and CD14 (HEK293-TLR4) were purchased from InvivoGen (San Diego, CA). HEK293 cells stably transfected with TLR2 (HEK293-TLR2) or TLR8 (HEK293-TLR8) were a gift from Dr. K. Fitzgerald (Worcester, MA). Cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% (v/v) FCS, 10 µg/ml Ciproflaxin and 2 mM L-glutamine. Selection agents were used as follows: HEK293-TLR4 cells, 10 µg/ml Blasticidin (Sigma) and 50 µg/ml of HygroGold (Invivogen); HEK293-TLR2 cells, 1 mg/ml G-418 (Sigma), HEK293-TLR8 cells, 10 µg/ml Blasticidin.

Receptor agonists - Ultrapure LPS from Escherichia coli (99.9% pure in respect to contaminating protein, DNA, and TLR2 agonists) was purchased from Alexis Biochemicals (Plymouth Meeting, PA). IL-1α was obtained from the National Cancer Institute (Frederick, MD). N-palmitoyl-S-dipalmitoylglyceryl Cys-Ser-(lys)₄ (PamCSK₄), macrophage-activating lipopeptide-2 (MALP2) and CL075 were purchased from Invivogen (San Diego, CA).

Antibodies - Mouse anti-c-Myc mAb (clone 9E10) and anti Flag M2 antibodies were purchased from Sigma. Mouse anti-α mAb was purchased from Covance (Princeton, NJ). Antibodies against GST-A46, encoded by a plasmid synthesised by inserting full length A46 downstream of GST in the bacterial expression vector pGEX4T2 were raised as described (32). The tumor necrosis factor receptor associated factor 6 (TRAF6) rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology. Anti-AU1 mAb was purchased from Eurogentec (Seraing, Belgium).

Plasmids - Sources of expression plasmids were as follows: pCMV-myc empty vector (Clontech), phRL-TK vector (Promega), pFR-luciferase and IFN-stimulated response element (ISRE)-luciferase reporter gene constructs (Stratagene), Gal4-IRF3, Gal4-IRF7, Flag-TRAM, Flag-TRAM P116H (TRAM PH), Flag-TRAM C117H (TRAM CH), and Flag-Mal (K.A. Fitzgerald, The University of Massachusetts Medical School, Worcester, MA), AU1-MyD88 and Myc-IL-1 receptor associated kinase 2 (IRAK2, M. Muzio, Mario Negri Institute, Milan, Italy), Flag-TRIF (S. Sato, Research Institute for Infectious Diseases, Osaka University, Japan), Myc-MyD88, AU1-MyD88 P200H (MyD88 PH), HA-Mal, HA-Mal P125H (Mal PH), and the glutathione S-transferase (GST) fusions of Mal, MyD88, TRAM, TIR-TRIF, TIR-TLR2, TIR-TLR3, TIR-TLR4, and TIR-TLR4 P712H (TLR4 PH, L.A. O’Neill, Trinity College Dublin, Dublin, Ireland), HA-TLR4 (J.V. McCarthy, University College Cork, Cork, Ireland), the NFκB reporter gene (described in (40)) and Flag-TRAF6 (Tularik, San Francisco, CA). The VACV open reading frame (ORF) A46 was previously cloned by PCR amplification from Western Reserve (WR) strain VACV DNA (40). Myc-A46ΔVIPER was generated by GenScript Corporation, Piscataway, NJ and contained the following mutations: K88D, F91S, K92D, L93G, I94G and L95G. Myc-A46 and GST-A46 were synthesised by inserting full-length A46 into the mammalian expression vector pCMV-MYC and the bacterial expression vector pGEX4T2 respectively.

Recombinant VACV viruses – VACV Western Reserve (vWT) and a VACV mutant (strain WR) lacking 93.5% of the A46 gene (vΔA46) have been previously described (32).

Reporter gene assays - HEK293-TLR4 cells (4 x 10⁴ cells per well) were seeded into 96-well plates and transfected 24 h later with expression vectors and luciferase reporter genes using GeneJuice (Novagen). In all cases, 20 ng/well of phRL-TK reporter gene was cotransfected to normalize data for transfection efficiency. The total amount of DNA per transfection was kept constant at 230 ng by addition of pCMV-Myc. Unless otherwise indicated 100 ng of A46 or A46ΔVIPER were used. After 24 h, cells were stimulated with 100 ng/ml LPS. After a further 8 h, cells were lysed.
in Passive Lysis Buffer (Promega) and whole cell lysates were analysed for luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity, and data are expressed as the mean fold induction, relative to control levels, for a representative experiment from a minimum of three separate experiments, each performed in triplicate. For the NFkB and ISRE assays, 60 ng of either κB-luciferase or ISRE-luciferase reporter genes were used. For the IRF3 and IRF7 assays, IRF3-Gal4 and IRF7-Gal4 fusion vectors (3 ng) were used in combination with 60 ng pFR luciferase reporter as described previously (32). The RANTES promoter assay was carried out using 60 ng of the RANTES promoter luciferase reporter gene.

Preparation of GST-fusion proteins - Empty pGEX.4T2 or pGEX.4T2 plasmid containing A46, Mal, MyD88, or the TIR domains of TRIF, TLR2, TLR3, TLR4 or TLR4 PH were transformed into Escherichia coli Rosetta-Gami B Host Strains (Novagen, Darmstadt, Germany) and grown in LB Broth. Protein expression was induced with 0.5 mM IPTG at 18°C for GST-TLR4 and 30°C for all others. Bacterial cells were harvested by centrifugation after 6 h induction (18 h in the case of GST-TLR4) and lysed by sonication in low-salt extraction buffer (300 mM NaCl, 1% Triton X-100, PBS). Insoluble fractions were removed by centrifugation. The remaining soluble fractions were cleared by glutathione sepharose 4B affinity chromatography (Amersham Biosciences) and levels of protein expression confirmed by SDS-PAGE and Coomassie staining of the gel.

GST pulldown assays - HEK293T cells were seeded into 15-cm dishes (3 x 10^6 cells) 24 h before transfection with GeneJuice. Cells were transfected with the relevant signaling molecule along with indicated amounts of A46. The total amount of DNA per transfection was kept constant by addition of pCMV-myc plasmid. Cells were harvested after 48 h in 850 µl lysis buffer (50 mM Hepes, pH 7.5, 250 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40 containing 0.01% aprotinin, 1 mM sodium orthovanadate, and 1 mM PMSF) for 30 mins on ice. For assessment of pulldown of HA–TLR4, the immune complexes were precipitated, washed four times in lysis buffer. For assessment of pulldown of HA–TLR4, the immune complexes were washed three times in wash buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.6), 0.1% Brij, 0.1% n-octyl-β-D-glucoside, 0.1% Triton-X-100). Pulldowns were analyzed by SDS-PAGE and immunoblotting.

For assessment of the effect of VACV-expressed A46 on interactions, HEK293T cells were infected with VACV vWT (wild type virus) or vΔA46 (virus lacking the A46 R gene) 24 h after transfection for 90 mins at 37°C, at a multiplicity of infection (MOI) of 1. The virus inoculum was aspirated and cell monolayers were overlaid with 5% FCS DMEM. Cells were harvested 24 h after infection in lysis buffer and GST pulldowns carried out as described. Immunoprecipitation and Immunoblotting - HEK293T cells were transfected and harvested as described for GST Pulldown assays. For co-immunoprecipitations, 4 µg of each construct was transfected. The total amount of DNA per transfection was kept constant by addition of pCMV-myc plasmid. For all co-immunoprecipitations, the appropriate antibodies, along with either protein A or protein G–Sepharose (Sigma), were incubated with the lysates for 2 h at 4°C. The immune complexes were precipitated, washed four times in lysis buffer, and analyzed by SDS-PAGE and immunoblotting.

Statistical analysis - Statistical analysis was carried out using paired Students t test.

RESULTS

A46 impairs ligand-induced TLR4-dependent transcription factor activation - We previously showed that VACV protein A46 inhibited signals induced by overexpression of the constitutively active TLR4, CD4–TLR4, such as NFκB, p38, ERK and IRF3 (32). To confirm similar effects on more physiologically relevant ligand-induced TLR4 complex activation, the effect of A46 on LPS-dependent human TLR4 activation was determined. To do this, transcription factor activation in HEK293 cells stably expressing
TLR4, CD14 and MD2 was measured by reporter gene assay (32,41), following LPS treatment of cells transfected with an A46 expression plasmid, or an empty vector. This showed that expression of A46 potently blocked ligand dependent activation of NFκB, IRF3 and IRF7 by TLR4 (Fig. 1A-C). LPS-induced activation of the RANTES (CCL5) and IFN-β promoters was also impaired by A46 (data not shown).

The conserved BB loop proline in TIR proteins is essential for interaction with A46 - A46 has been previously shown to interact with components of the TLR4 signalling complex containing TIR domains, namely TLR4 itself, Mal, MyD88, TRIF and TRAM (32), and it is through these interactions that A46 can likely exert its inhibitory effects. However, how exactly A46, a protein predicted to have a bcl-2-like fold (38) (42), can interact with and antagonise TIR proteins is unclear. We therefore analysed the ability of A46 to interact with a range of TIR proteins, with a view to obtaining further information on the basis for such interactions. For this, a GST pulldown assay was used, whereby the ability of A46 expressed in cells to interact with GST fusions of TLR TIR domains was examined. Fig. 2A shows that A46 interacted with the TLR2 and TLR4 TIR domains, but not the TLR3 TIR domain (lanes 2-4), nor with GST alone (lane 6). Coupled to this, we previously demonstrated that A46 does not interact with SARM (32). The TIR domains of both SARM and TLR3 differ from those of TLR2 and TLR4 in that instead of the conserved proline in the BB loop, there is an alanine. Therefore, we wondered whether A46 required an intact proline in the BB loop of TIR proteins for interaction. A proline to histidine mutation in the BB loop region of TLR4 renders C3H/HeJ mice unresponsive to LPS (29). When this mutation was introduced into the GST fusion of the TIR domain of TLR4 (P714H), A46 could no longer interact with this protein (Fig. 2A, lane 5). Thus for TLR proteins, the presence of a BB loop proline is predictive of an ability to interact with A46.

In order to test whether this was also the case for the interactions between A46 and the TLR4 adaptors, the corresponding BB loop proline in MyD88, Mal, TRIF and TRAM was mutated, and the ability of wild type and mutant adaptor proteins expressed in HEK 293 cells to co-immunoprecipitate with A46, and to interact with GST-A46, was examined. A46 associated with wild-type MyD88 (Fig. 2B and 2F, left panels), but not with MyD88 P200H (Fig. 2B and 2F, right panels). Similarly, although A46 interacted robustly with wild type Mal, this association was lost for Mal P125H (Fig. 2C and 2F). TRAM contains a cysteine residue at the site of the conserved BB loop proline but also has a proline directly adjacent to the cysteine. When either of these residues was mutated to histidine (TRAM P116H or TRAM C117H), A46 was unable to interact with TRAM in both co-immunoprecipitation (Fig. 2D) and GST-pulldown experiments (Fig. 2F) even though the A46-TRAM association was clearly visible under the same experimental conditions (Fig. 2D and 2F). Similarly, A46 interacted with wild-type TRIF, but could not do so with TRIF P434H (Fig. 2E and 2F).

Consistent with the requirement for an intact TIR BB loop proline for TIR adaptor:A46 binding, A46 lost its ability to inhibit MyD88 or TRIF function when their BB loop proline was mutated to histidine. Supplemental Fig. 1A shows that transfection of either wild type MyD88 or MyD88 P200H into cells led to activation of NFκB. Co-transfection with increasing amounts of A46 led to dose-dependent inhibition of the MyD88-dependent signal, but had no effect on MyD88 P200H-dependent NFκB activation (Suppl. Fig. 1A). Similarly, A46 inhibited TRIF- but not TRIF P434H-induced NFκB (Suppl. Fig. 1A) and IRF3 (Suppl. Fig. 1B) activation. These experiments were not possible for Mal P125H, TRAM P116H or TRAM C117H since these mutated proteins lose the ability to drive transcription factor activation (43) (44) (41), which further attests to the importance of the TIR BB loop domain for proper function.

These data show that the ability of A46 to interact with multiple TIR domain-containing proteins is in each case dependent on such proteins having a proline residue in the BB loop domain, and that A46 only inhibits signaling by adaptors with intact BB loops, consistent with the notion that the BB loop motif has been selected by poxviruses for antagonism.

A46 disrupts receptor:adaptor, and not receptor:receptor nor adaptor:adaptor, interactions in the TLR4 complex – Evidence suggests that the BB loop motif is critical for TIR function, and for at least some TIR:TIR interactions (see Introduction). Since A46 targets intact BB loop motifs of TIR proteins for interaction with components of the TLR4...
complex (TLR4, MyD88, Mal, TRAM and TRIF), it seemed likely that its mechanism of inhibition of TLR4 would involve disruption of some or all of the TIR:TIR interactions required for intact signalling. These can be classified as receptor:receptor (TLR4:TLR4), receptor:adaptor (TLR4:Mal and TLR4:TRAM) and adaptor:adaptor (Mal:MyD88 and TRAM:TRIF) interactions. Thus competition GST-pulldown assays were used to investigate the effects of A46 on these TIR protein interactions. To assess the effect of A46 on the TLR4:TLR4 TIR interaction, cells were co-transfected with TLR4 and an increasing amount of A46 plasmid. Lysates were then incubated with equal amounts of either GST or GST-TLR4 TIR domain (Fig. 3A). As expected, GST-TLR4 TIR domain pulled down HA-TLR4 (Fig. 3B, top panel, lane 1), but GST alone could not (Fig. 3B, fourth panel, lane 1). The presence of increasing amounts of A46 had little effect on the ability of GST-TLR4 TIR domain to pull down HA-TLR4 (Fig. 3B, top panel, lanes 2, 3 and 4). Although some variability in the intensity of the TLR4-TLR4 interaction bands in all lanes is observed due to the difficulty of immunoblotting for TLR4 (Fig. 3B, top panel), there is still an interaction band visible at the highest dose of A46 (lane 4), suggesting that A46 does not impair the receptor:receptor interaction in the TLR4 complex. In contrast, using the same experimental set up, A46 could be seen to strongly inhibit TLR4:adaptor interactions: the presence of increasing amounts of A46 significantly impaired the ability of GST-TLR4 TIR domain to pull down Mal (Fig. 3C, top panel, compare lanes 3 and 4 to lane 1). This observed impairment was not due to unequal amounts of Mal present in the different samples (Fig. 3C, third panel). A46 had an even more dramatic effect on the TLR4:TRAM interaction, where even the lowest dose of A46 plasmid severely affected the ability of GST-TLR4 TIR to interact with TRAM (Fig. 3D, top panel, compare lanes 2-4 to lane 1). Again, equal expression levels of TRAM were observed in all experimental samples (Fig. 3D, third panel). These data show that A46 can disrupt receptor:adaptor interactions in the TLR4 complex.

To assess whether A46 can impair the TLR4-Mal or TLR4-TRAM associations in the context of VACV infection, HEK293T cells transfected with either HA-Mal (Fig. 3E) or Flag-TRAM (Fig. 3F) were co-transfected with myc-A46, infected with wild type VACV expressing A46 (vWT) or infected with virus where the gene encoding A46 (A46R) was absent (vΔA46). Similar levels of A46 expression were observed when cells were transfected with myc-A46 or infected with VACV vWT (Figs. 3E and 3F, second panel, lanes 2 and 3). Although vΔA46 viruses expressed no A46 as expected, they did express A52 at similar levels to wild type virus ((Figs. 3E and 3F, fourth panel, lanes 3 and 4)). When GST pulldowns were performed as before, the TLR4:Mal and TLR4:TRAM interactions were impaired in cells infected with vWT (Fig 3E and 3F, compare lane 3 to lane 1), or in cells expressing A46 (lane 2). Compellingly, there was less impairment of the TLR4:Mal and TLR4:TRAM interactions in cells infected with vΔA46 compared to cells infected with vWT (lane 3). These data show that VACV infection is capable of impairing TLR4-Mal and TLR4-TRAM associations and that virally expressed A46 is largely responsible for disrupting these interactions.

We next used to GST pulldown system to investigate the effect of A46 on the adaptor:adaptor interactions in the TLR4 complex, i.e. the associations between Mal, MyD88 and TRIF-TRAM. When GST-Mal was used to pull down MyD88 in the presence of increasing amounts of A46 no impairment of the Mal-MyD88 interaction was observed, even at the highest dose of A46 (Fig. 4A, top panel). Similarly, A46 could not impair the interaction between GST-TRIF TIR and TRAM (Fig. 4B, top panel). The crystal structure of Mal has recently been determined, which suggested that the physiologically relevant form of Mal for signalling may be a dimer (22) (23). Thus, in determining the effect of A46 on adaptor:adaptor interactions relevant to TLR4 complex function, we also tested the ability of A46 to disrupt the Mal:Mal interaction, and found no effect of A46 on this (Fig. 4C). Thus under identical experimental conditions where receptor:adaptor interactions were impaired by A46 (TLR4:Mal, TLR4:TRAM), A46 had no effect on adaptor:adaptor associations (Mal:MyD88, TRAM:TRIF, Mal:Mal).

We also tested whether A46 binding to adaptor proteins affected their interaction with downstream signalling proteins. Previous studies have implicated IRAK-2 in IL-1R and TLR4 signalling, and shown that IRAK-2 can associate with MyD88 and Mal (43,45) (46) (47). Here,
either GST-Mal or GST-MyD88 could pull down IRAK2 from cell lysates, but A46 was unable to significantly impair these associations (Fig. 4D, first and second panels). Studies have shown that Mal and TRAF6 directly interact in response to TLR4 stimulation (48) (49), and hence the effect of A46 on the Mal-TRAF6 interaction was also assessed. Similar to the case for MyD88:IRAK2 and Mal:IRAK2, the Mal:TRAF6 interaction was unimpaired by A46 (Fig. 4E, top panel). Altogether, these data show that for the TLR4 complex, A46 targets receptor:adaptor, and not receptor:receptor, adaptor:adaptor nor adaptor:effector interactions for antagonism. 

The ability of A46 to antagonise TIR:TIR interactions correlates with a requirement for an intact adaptor BB loop for these interactions – Since A46 targets TIR BB loop motifs, and interferes with receptor:adaptor but not adaptor:adaptor interactions, we reasoned that the adaptor BB loop motif would be required for the former but not the latter type of interactions within the TLR4 complex. Thus to examine the importance of the adaptor BB loop in receptor:adaptor associations, the interactions between TLR4 and Mal, Mal P125H, TRAM, TRAM C116H or TRAM P117H were assessed. GST-TLR4 was capable of pulling down Mal (Fig. 5A, top panel, lane 1), TRAM (Fig. 5B, top panel, lane 1), TRAM P117H (Fig. 5B, top panel, lane 2), but was impaired in its ability to pull down Mal P125H (Fig 5A, top panel, lane 2) or TRAM C117H (Fig. 5B, top panel, lane 3). Thus the Mal and TRAM BB loop regions are required for receptor:adaptor interactions. To assess the importance of the BB loop motifs in adaptor:adaptor interactions, the abilities of Mal and MyD88 to interact were compared with those of Mal P125H and MyD88 P200H. Fig. 5C shows that Mal and MyD88 can co-immunoprecipitated (Top panel, lane 3), which was also apparent for Mal P125H and MyD88 P200H (Fig. 5D, top panel, lane 3), as has been previously reported (16). Thus the adaptor:adaptor interaction between Mal and MyD88 is independent of their BB loop Prolines.

Hence there is a correlation between the TIR:TIR interactions that are antagonised by A46, and those that require an intact adaptor BB loop motif.

Identification of a TLR4-specific inhibitory motif in A46 – Based on the premise that immune inhibitory viral proteins have optimised surface motifs to maximally target host proteins by directly binding to them, we recently screened peptides derived from A46R in order to ascertain whether individual peptides would recapitulate any of the TLR inhibitory ability of the full-length protein. Intriguingly we found one such peptide, which we called VIPER (38). Although A46 can inhibit signalling by multiple TLR complexes (32), VIPER (containing the 11 amino acids KYSFKLILAEY from A46 fused to a cell-penetrating delivery sequence), potently and specifically inhibited TLR4 responses and was inert against other TLRs (38). Further, VIPER could pull down Mal or TRAM (but not MyD88 or TRIF) from cell lysates (38). Thus we wondered whether the region of A46 from which VIPER was derived represented the TLR4-specific inhibitory surface of A46, and whether disruption of this sequence would impair the ability of A46 to interact with TRAM and/or Mal. To test this, a portion of the sequence corresponding to VIPER in A46, KYSFKLIL, was mutated to DYSSDDGGG to generate A46ΔVIPER, which was expressed in cells at a similar level to A46 (Fig. 6A).

Remarkably, A46ΔVIPER selectively lost its ability to inhibit TLR4, and not other TLR pathways. In contrast to A46, A46ΔVIPER could no longer inhibit LPS-induced NFκB activation (Fig 6B) nor could it impair LPS-induced activation of the IRF3-dependent ISRE (Fig. 6C). However, A46ΔVIPER was equally as potent as A46 in inhibiting IL-1R-, TLR2- and TLR8-dependent NFκB activation (Figs. 6D, E and F). Interestingly, A46ΔVIPER failed to co-immunoprecipitate with TRAM, in contrast to A46 (Fig. 6G, top panel, lane 4), but still retained the ability to interact with Mal (Fig. 6G, top panel, lane 3). Consistent with this, A46ΔVIPER inhibited Mal- but not TRAM-mediated NFκB activation (Fig. 6H). Together, these data show that the region of A46 from which VIPER is derived is a TLR4-specific inhibitory surface that selectively targets TRAM, and not Mal, in the intact protein.

DISCUSSION

Although the viral or endogenous ligand that mediates the anti-poxviral TLR4 activity is yet to be discovered, the fact that TLR4 is protective in pulmonary vacv infection (9) provides a rationale as to why poxviruses such as VACV encode for the TLR4 inhibitor A46. A46 is expressed early during infection of cells, and VACV lacking A46 has been found to be less
virulent (32). However, how exactly A46 would antagonise TLR4 signalling remained to be discovered. Here we present data demonstrating that A46 prevents critical protein:protein interactions necessary for proper TLR4 signalling, and also identify motifs within the host proteins targeted, and within A46, required for the inhibition of TRAM, the TLR4-specific TIR adaptor.

A46 was originally identified on the basis of sequence similarity to TIR domains (40) (32). Therefore, it was assumed that A46 would adopt a TIR fold and bind to TIR proteins via homotypic interactions. However, the crystal structures of A52 and B14, poxviral proteins related to A46, have been determined and shown that the proteins adopt a bcl-2-like fold, (39). Subsequent biophysical analysis of the purified C-terminal region of A46 (residues 81-230) revealed a highly α-helical structure, consistent with a bcl-2 fold (42). Thus defining the mechanism of action of A46 became more challenging as, instead of being a viral mimic of a host TIR domain that could act as a dominant negative, it is likely a Bcl-2-like protein which targets TIR proteins in a manner not involving homotypic TIR-TIR interactions. It is not unprecedented for TIR proteins and Bcl-2-like proteins to interact, since TRIF and MyD88 can associate with both Beclin-1 and Bcl-2, and are thought to regulate autophagy by disrupting the Beclin1-Bcl2 interaction (50). A46 is part of a family of poxviral proteins either demonstrated or predicted to contain a bcl-2 fold, all of which inhibit PRR signaling, but do so by interacting with diverse and structurally dissimilar host proteins. Thus A52 targets IRAK-2 and TRAF6 ((33), (51), (46)), B14 interacts with IKKβ (35), K7 interacts with DDX3 (36) and C6 associates with TANK, NAP1 and SINTBAD (37). This is accomplished by each viral protein having, as well as the core bcl-2 fold, unique host protein interaction surfaces or loops.

Here we showed that A46 targets the conserved BB loop proline of TIR proteins and has no ability to associate with TLRs or TIR adaptors that lack this conserved proline residue. Further, A46 acted on the TLR4 complex by disrupting the TLR4-Mal and TLR4-TRAM interactions. We also demonstrated that these interactions were targeted by VACV during the course of viral infection. However, A46 had no effect on the TLR4-TLR4, Mal-MyD88 or TRAM-TRIF interactions. We found that the requirement for a BB loop for protein interface interactions correlated with the protein:protein interfaces antagonised by A46. Previous data indicates the interactions between TLR4 and Mal or TRAM requires the presence of the conserved proline in the BB loops of these proteins (16,44), and also cysteine in the case of TRAM (41). Consistent with Dunne et al. (16) here we found Mal P125H interacts with MyD88 P200H to the same extent as wt Mal associates with wt MyD88. A46 requires the presence of the conserved proline to associate with its TIR interaction partners, and only impairs associations where these BB loop prolines are required. Thus our data, together with previous work, altogether suggests that TIR BB loop Prolines are important only in receptor:adaptor interactions and not in adaptor:adaptor interactions.

The crystal structure of the TIR domain of Mal has recently been described (22) (23). These studies showed that the Mal-TIR fold differs from other TIR domain structures because it lacks a helical segment (αB) between the βB- and βC-strands and instead contains a long loop (AB) connecting the first helix (αA) and the βB-strand. This AB loop sequence, whose structure could not be fully determined likely due to inherent flexibility, shows significant sequence similarity with the functionally important BB loop segment in other TIR domains, suggesting that the sequence retains BB loop functions despite a structural rearrangement having occurred (22) (23). That the Mal AB loop is very similar to other TIR BB loops is consistent with the fact that A46 targets Mal, and requires Mal P125 to do so. Valkov et al. (22) used Mal mutagenesis, modelling and docking studies to suggest a scenario whereby the Mal:TLR4 interaction surface required the Mal AB loop, while the Mal:Mal and Mal:MyD88 interaction surfaces did not, consistent with our data here, and with the ability of A46 to only disrupt Mal:TLR4, and not Mal:Mal nor Mal:MyD88 interactions.

By preventing or disrupting TLR4:Mal and TLR4:TRAM interactions, A46 would be expected to prevent ligand-stimulated activation of the TLR4 complex, and disable downstream transcription factor activation, as is shown here (Figs 1 and 6). Although both Mal and TRAM are localised to the plasma membrane via a PIP2 binding motif (52) and lipid-raft localisation signal respectively (53), Mal-dependent signalling occurs at the plasma membrane via engagement of the MyD88-containing
myddosome leading to early NFκB activation while, TRAM-dependent signaling occurs from endosomes via engagement of TRIF leading to late NFκB, and IRF3 activation (52) (53). A model has recently been proposed whereby TRAM would be engulfed with the TLR4 complex when the receptor complex is endocytosed after plasma membrane Mal-dependent signaling, but would only properly engage with TLR4 once the receptor undergoes an endosome-specific conformational change, due to the lower pH of endosomes (18). Thus A46 likely antagonises TLR4 both at the plasma membrane (to disrupt Mal signaling) and at the endosome (to inhibit TRAM), either by preventing Mal being recruited to TLR4 at the membrane, and TRAM engaging properly with TLR4 at the endosome and/or displacing TRAM or Mal from activated TLR4 complexes.

In the case of the A46:TRAM interaction, we were able to identify the motif on A46 that targets TRAM. The VIPER peptide derived from A46 inhibits TLR4 (and not other TLRs) and associates with Mal and TRAM in cell lysates (38). When the region of A46 from which VIPER is derived was mutated to generate A46ΔVIPER, the ability of A46 to impair LPS-induced NFκB activation was lost, as was its ability to interact with TRAM and to inhibit TRAM-mediated NFκB activation. However A46ΔVIPER still associated with Mal and inhibited Mal-, IL-1R-, TLR2- and TLR8-dependent NFκB activation. The reason why VIPER peptide interacts with both Mal and TRAM in cell lysates, while A46ΔVIPER only loses its ability to interact with TRAM and not Mal remains to be resolved, but is consistent with our previous suggestion that A46 does not have a generic interaction site for all the TIR proteins it antagonises, but rather different regions of A46 are specific for interaction with different proteins (38). Thus in the intact protein, the VIPER motif represents the region of A46 that specifically interacts with TRAM. It is unlikely that the conformation of the isolated VIPER peptide is identical to the predicted three-dimensional epitope formed by this segment in intact A46, which may partly explain the difference. Very interestingly, the monkeypox A46 protein has a substitution of a proline instead of a leucine in the VIPER motif, and monkeypox A46 inhibits Mal- but not TRAM-dependent NFκB activation (data not shown), consistent with the VIPER motif being particularly important for TRAM antagonism. Furthermore, since viruses optimally target residues and motifs in host proteins that are critical for function, future studies may reveal novel sites on TRAM to target therapeutically.

Overall this study provides the molecular basis for poxviral subversion of TLR4 signalling and clarifies the importance of TIR motif BB loops in the formation of the TLR4 complex.

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**FOOTNOTES**

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4Abbreviations used: IRAK, IL-1 receptor associated kinase; IRF, Interferon regulatory factor; ISRE, Interferon-stimulated response element; Mal, MyD88-adaptor like; MyD88, Myeloid differentiation primary response gene 88; NFkB, Nuclear factor kappa B; PAMP, Pathogen associated molecular pattern; PRR, Pattern recognition receptor; SARM, sterile α and HEAT-armadillo motif protein; TIR, Toll/IL-1R; TLR, Toll-like receptor; TRAF, tumor necrosis factor receptor associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain containing adaptor protein inducing IFN-β; VACV, Vaccinia Virus; VIPER, Viral inhibitory peptide of TLR4.

**FIGURE LEGENDS**

Figure 1: A46 impairs ligand-induced TLR4-dependent transcription factor activation. HEK293-TLR4 cells were transfected for 24 h with 150 ng myc-A46 or pCMV-myc (EV) and the NFkB luciferase reporter gene (A), or the pFR luciferase reporter gene along with either IRF3-Gal4 (B) or IRF7-Gal4 (C). Cells were stimulated with 10 ng/ml LPS for 6 h and luciferase reporter gene activity was measured. The data are mean ± SD of triplicate samples and are representative of at least five separate experiments. *p<0.05, **p<0.005 or ***p<0.0005 compared to LPS and EV.

Figure 2: The conserved Proline of the BB loop is essential for A46 to interact with the TIR adaptors. A, HEK293T cells were transfected with 4 µg myc-A46. After 48 h, lysates were incubated with GST-TLR TIR domains or GST alone as indicated, and together with input lysate (lane 1), were analyzed by SDS-PAGE and immunoblotting. GST and GST-fusion proteins were analysed by SDS-PAGE and Coomassie staining to demonstrate equal inputs. B-E, HEK293T cells were transfected with A46 and (B) AU1-MyD88 (left) or AU1-MyD88 P200H (right), (C) HA-Mal (upper panel) or HA-Mal P125H (lower panel), (D) Flag-TRAM (upper panel), Flag-TRAM P116H (middle panel) or Flag-TRAM C117H (lower panel) and (E) Flag-TRIF (upper panel) or Flag-TRIF P434H (lower panel).
as indicated. After 48 h, lysates were subject to immunoprecipitation, SDS-PAGE, and immunoblotting with the indicated antibodies. F, HEK293T cells were transfected with 8 µg of the indicated TIR adapter molecules. After 48 h, lysates were incubated with GST alone (lane 2) or GST-A46 (lane 3), and together with input lysates (lane 1) were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. Each immunoblot is representative of three experiments (A-F).

Figure 3: A46 inhibits TLR4 signalling by disrupting receptor:adaptor, and not receptor:receptor interactions in the TLR4 complex. A, Coomassie-stained gel showing equal amounts of GST and GST-TLR4 TIR. B-D, HEK293T cells were transfected with the indicated amounts of HA-TLR4 (B), HA-Mal (C) or Flag-TRAM (D), along with increasing amounts of myc-A46 or pCMV-myc and harvested after 48 h. E-F, HEK293T cells were transfected with HA-Mal (E) or Flag-TRAM (F), along with either myc-A46 or pCMV-myc. After 24 h, cells were infected with VACV vWT or vΔA46 (MOI = 1) as indicated. Cells were harvested 24 h after infection. B-F, Clarified lysates were incubated with GST alone or GST-TLR4 TIR and, together with input lysates, were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. Each immunoblot is representative of at least three experiments.

Figure 4: A46 does not disrupt adaptor:adaptor interactions in the TLR4 complex. A-E, HEK293T cells were transfected with the indicated amounts of myc-MyD88 (A), Flag-TRAM (B) HA-Mal (C), myc-TRAK2 (D) or Flag-TRAF6 (E), along with increasing amounts of myc-A46 or pCMV-myc. After 48 h, lysates were incubated with GST alone or GST-fusion protein and, together with input lysates, were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. Each immunoblot is representative of at least three experiments.

Figure 5: Receptor:adaptor but not adaptor:adaptor interactions require intact adaptor BB loops. A-B, HEK293T cells were transfected with 4 µg HA-Mal, HA-Mal PH (A) or Flag-TRAM, Flag-TRAM PH and Flag-TRAM CH (B). After 48 h, lysates were incubated with GST-alone or GST-TLR4 TIR, and together with input lysates, were analyzed by SDS-PAGE and immunoblotting. (C-D) HEK293T cells were transfected with 4 µg HA-Mal and AU1-MyD88 (C) or 4 µg HA-Mal PH and AU1-MyD88 PH (D). After 48 h, lysates were subject to immunoprecipitation, SDS-PAGE, and immunoblotting with the indicated antibodies. Each immunoblot is representative of at least three experiments.

Figure 6: The region of A46 from which the VIPER peptide is derived is required for TLR4 inhibition and TRAM binding. A, HEK293-TLR4 cells were transfected with 2 µg of pCMV-myc, myc-A46 or myc-A46ΔVIPER. After 24 h, clarified lysates were subject to SDS-PAGE and immunoblotting with the anti-myc antibody. B-C, HEK293-TLR4 cells were transfected with pCMV-myc (EV), 50-150 ng myc-A46 or 50-150 ng myc-A46ΔVIPER, and the NFkB (B), or the ISRE (C) luciferase reporter gene. Cells were stimulated with 10 ng/ml LPS for 6 h and luciferase reporter gene activity was measured. The data are mean ± SD of triplicate samples and are representative of at least four separate experiments. D-F, HEK293 cells stably expressing the IL-1 receptor (D), TLR2 (E) or TLR8 (F) were transfected with pCMV-myc (EV), 50-150 ng myc-A46 or 50-150 ng myc-A46ΔVIPER, and the NFkB luciferase reporter gene. Cells were stimulated with either 50 ng/ml IL-1α (D), 20 nM MALP2 (E), 20 ng/ml Pam3CSk4 (E) or 2.5µg/ml CL075 (F) for 6 h and luciferase reporter gene activity was measured. The data are mean ± SD of triplicate samples and are representative of at least four experiments. G, HEK293T cells were transfected with 4 µg of Flag-Mal or Flag-TRAM along with either myc-A46 or myc-A46ΔVIPER. After 48 h, lysates were subject to immunoprecipitation, SDS-PAGE, and immunoblotting with the indicated antibodies. Each immunoblot is representative of three experiments. H, HEK293T cells were transfected with 25 ng Flag-Mal or Flag-TRAM and 50-150 ng A46, 50-150 ng A46ΔVIPER or pCMV-myc (EV), along with the NFkB luciferase reporter gene. The data are presented as percentage inhibition of fold induction and are representative of three experiments. *p<0.05 or **p<0.005 compared to stimulus only.
Figure 1

A  

NfkB

Fold Induction

EV  A6  EV  A6

LPS

***

B  

IRF3

Fold Induction

EV  A6  EV  A6

LPS

**

C  

IRF7

Fold Induction

EV  A6  EV  A6

LPS

*
Figure 4

A) myc-MyD88 (µg) 5 5 5 5 0 5 10 15
myc-A46 (µg) 1 2 3 4
Pulldown with GST-Mal
IB: myc
IB: myc
IB: myc
Pulldown with GST
IB: myc

B) Flag-TRAM (µg) 5 5 5 5
myc-A46 (µg) 0 5 10 15
Pulldown with GST-TRIF
IB: Flag
IB: myc
IB: Flag
Pulldown with GST
IB: Flag

C) HA-Mal (µg) 5 5 5 5
myc-A46 (µg) 0 5 10 15
Pulldown with GST-Mal
IB: HA
IB: myc
IB: HA
Pulldown with GST
IB: HA

D) myc-IRAK2 (µg) 6 6 6 6
myc-A46 (µg) 0 3.5 7 14
Pulldown with GST-Mal
IB: myc
IB: myc
IB: myc
Pulldown with GST
IB: myc

E) TRAF6 (µg) 3 3 3 3 0 2.5 7.5 12.5
myc-A46 (µg) 1 2 3 4
Pulldown with GST-Mal
IB: TRAF6
IB: myc
IB: TRAF6
Pulldown with GST
IB: TRAF6
Figure 5

A

Pulldown with GST-TLR4
IB: HA
IB: HA

B

Pulldown with GST-TLR4
IB: Flag
IB: Flag
Pulldown with GST
IB: Flag

C

HA-Mal + - +
AU1-MyD88 - + +
IP: HA
IB: AU1
IP: HA
IB: HA
IB: HA
IB: AU1

1 2 3

D

HA-Mal PH + - +
AU1-MyD88 PH - + +
IP: HA
IB: AU1
IP: HA
IB: HA
IB: HA
IB: AU1

1 2 3
**Supplemental Figure 1:** The conserved Proline of the BB loop is essential for A46 to exert its inhibitory effects. HEK293T cells were transfected with 25 ng AU1-MyD88, AU1-MyD88 PH, HA-TRIF or HA-TRIF PH, 50 or 100 ng A46 or pCMV-myc (EV), along with the NFκB luciferase reporter gene (A), or the pFR luciferase reporter gene and IRF3-Gal4 (B). The data are presented as percentage inhibition of fold induction and are representative of at least three experiments.