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Identification of a second binding site on the TRIM25 B30.2 domain

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Abstract

The retinoic acid-inducible gene-I (RIG-I) receptor recognizes short 5'-di- and triphosphate base-paired viral RNA and is a critical mediator of the innate immune response against viruses such as influenza A, Ebola, HIV and hepatitis C. This response is reported to require an orchestrated interaction with the tripartite motif 25 (TRIM25) B30.2 protein-interaction domain. Here, we present a novel second RIG-I-binding interface on the TRIM25 B30.2 domain that interacts with CARD1 and CARD2 (caspase activation and recruitment domains) of RIG-I and is revealed by the removal of an N-terminal α -helix that mimics dimerization of the full-length protein. Further characterization of the TRIM25 coiled-coil and B30.2 regions indicated that the B30.2 domains move freely on a flexible tether, facilitating RIG-I CARD recruitment. The identification of a dual binding mode for the TRIM25 B30.2 domain is a first for the SPRY/B30.2 domain family and may be a feature of other SPRY/B30.2 family members.

Introduction

Viral infections cause profound morbidity and mortality and remain a significant global health issue. The first line of defense against infection is mediated by the host innate immune response. Viral pathogens and/or their products are, in general, detected by three families of invariant pathogen recognition receptors: the membrane-bound Toll-like receptors, the cytoplasmic, retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and the DNA sensor, cyclic guanosine monophosphate-adenosine mono-phosphate synthase (cGAS) [1,2].

The RLRs consist of the prototypic member RIG-I, MDA5 (melanoma differentiation-associated gene 5) and LGP2 (Laboratory of Genetics and Physiology gene 2), all of which

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

contain a DExD/H box RNA helicase/ATPase domain and a C-terminal domain (CTD). RIG-I and MDA5 contain two N-terminal caspase activation and recruitment domains (CARDs), which mediate downstream signaling via MAVS aggregation (mitochondrial anti-viral signaling; also known as IPS-1/Cardif/VISA), which is localized to the outer mitochondrial membrane [3–7]. The RIG-I CTD binds to viral 5' diand triphosphate-containing ds/ssRNA in the cytoplasm of infected cells, with binding specificity determined by the conformation of the RNA-binding loop within the CTD [8–10]. Upon RNA binding and hydrolysis of ATP, RIG-I undergoes a major conformational change that releases the CARDs for dephosphorylation by the protein phosphatases PP1 α and γ and, subsequently, TRIM25 binding [11–15].

TRIM proteins are characterized by a RING/B-box/coiled-coil core, approximately half of which contain an SPRY/B30.2 domain at the C-terminus [16]. The RING domain confers E3 ligase activity, whereas the B30.2 domain is responsible for binding the substrate molecule. The function of the B-box domain is largely unknown and the coiled-coil domain (CCD) is required for dimerization/oligomerization [16] (Figure 1a).

The importance of TRIM25 in the RIG-I-mediated response to infection is illustrated by *Trim25*^{-/-} mouse embryonic fibroblasts, which have a profound defect in IFN- β production and restriction of viral replication [17]. TRIM25 has been shown to interact via its B30.2 domain with the first CARD of RIG-I (CARD1) and then synthesize K63-linked polyubiquitin chains, some of which are covalently attached to lysines in the second CARD (CARD2) of RIG-I, while others are non-covalently attached to both CARDs [18–20]. The ubiquitination is thought to promote tetramerization of the RIG-I CARDs, which in turn facilitates MAVS aggregation on the mitochondrial membrane [4,19]. MAVS acts as a recruitment platform for the assembly of two kinase-containing complexes that activate NF- κ B and IRF3/7 to promote the expression of proinflammatory cytokines and, in particular, induce transcription of type I IFN [21].

A RIG-I-independent role for TRIM25 in the host anti-viral response has also recently been described. Meyerson et al. [22] have shown that TRIM25 can bind viral ribonucleoprotein complexes and block viral replication, and the RNA-binding activity appears to be mediated by both CCD and B30.2 domains [23].

We have previously solved a high-resolution crystal structure of the TRIM25 B30.2 domain, and characterized a RIG-I-binding site, which corresponds spatially to known interaction sites on the TRIM21 and SPSB2 SPRY/B30.2 domains [24]. Here, we identify a novel second binding site on the TRIM25 B30.2 domain. These observations have broader implications for understanding other SPRY/B30.2 domain interactions, and in particular, the role of other TRIM proteins such as TRIM5 α and Pyrin, in innate immunity.

Results

Deletion of the TRIM25 α 1 helix reveals a novel RIG-I CARD-binding site

Previous studies have shown a robust interaction between the TRIM25 B30.2 domain (residues 455–634) and the RIG-I CARDs [18,25]. Our identification of a RIG-I-binding site

on the TRIM25 B30.2 domain (site 1; Figure 1b) was confirmed by immunoprecipitation studies, which yielded a surprisingly weak interaction between the TRIM25 B30.2 domain (residues 440–634) and the RIG-I CARDS [24]. Differences in the length of the constructs used suggested that deletion of the 15 residues at the N-terminus of the B30.2 domain may result in a stronger interaction with the RIG-I CARDS. These residues corresponded to the α 1 helix in our TRIM25 B30.2 domain structure, located on the opposing face of the domain from the previously characterized RIG-I-binding site 1 [24]. According to the 3D structure of the TRIM25 B30.2 domain, the α 1 helix could restrict access to the underlying residues (site 2; Figure 1b).

To determine if the α 1 helix was indeed obscuring a second binding site on the TRIM25 B30.2 domain, HEK293T cells were transfected with FLAG-tagged α 1-containing and α 1-deleted (α 1 Δ), human and mouse TRIM25 B30.2 constructs and tested for their ability to interact with MYC-tagged human and mouse RIG-I CARD domains (2CARD; residues 1–200). A FLAG-tagged SPSB2 construct (SprA/Ryanodine receptor domain and SOCS Box containing 2) was used as a negative control. TRIM25 B30.2 complexes were recovered with anti-FLAG antibodies and assayed for bound RIG-I 2CARD by Western blotting. The α 1 constructs interacted strongly with human and mouse RIG-I 2CARDS, in stark comparison to the lack of interaction observed with the α 1-containing TRIM25 B30.2 domains or the SPSB2 negative control (Figure 2a).

To further confirm the specificity of the RIG-I CARD interaction with TRIM25 B30.2 α 1, constructs expressing, either FLAG-tagged mouse or human RIG-I 2CARD, the SPSB2 SPRY domain, MAVS or MAVS CARD were transfected into HEK293T cells and assessed for their ability to interact with MYC-tagged mouse TRIM25 B30.2 α 1. TRIM25 B30.2 α 1 again interacted strongly with RIG-I 2CARD, but not with the SPSB2 SPRY domain, MAVS or MAVS CARD (Figure 2d), suggesting that the observed interaction between TRIM25 B30.2 α 1 and RIG-I CARDS was specific and not an artifact of the system, nor simply a result of exposing a hydrophobic region on the B30.2 domain.

To investigate which residues in the exposed β -sheet face (putative site 2) might be important for RIG-I binding, a series of FLAG-tagged TRIM25 B30.2 alanine mutants were tested for binding to MYC-tagged mouse RIG-I 2CARD. When compared with the wild-type B30.2 α 1, the single mutants, F592A and L604A, showed consistently decreased binding to the RIG-I CARDS, with the most dramatic decrease in binding observed with the I594A/L604A double mutant. H590A and I594A alone had a minor effect on binding, while no effect was seen with N587A (Figure 2b). The mutational analysis suggests that F592, I594 and L604 are key TRIM25 B30.2 residues, probably to be involved in binding to RIG-I 2CARD. This is further supported by conservation of these residues in TRIM25 from other species. H590, F592 and I594 are not conserved in other SPRY/B30.2 domains, suggesting that these site 2 residues are involved in TRIM25-specific interactions (Supplementary Figure S1c).

Based on our structural data, L446 and L450 in the α 1-helix co-ordinate many contacts with the underlying β -sheet (Figure 1b), predicting that disruption of these contacts may allow the RIG-I 2CARDS access to site 2, even when α 1 is present. Accordingly, these residues were

mutated to arginine and the constructs were tested for binding to RIG-I 2CARDs. Consistent with previous attempts, the mouse α 1-containing TRIM25 B30.2 domain showed no detectable interaction with the mouse RIG-I 2CARDs. However, mutation of the B30.2 L446 to arginine dramatically increased the interaction of the α 1-containing B30.2 domain with mouse RIG-I (Figure 2c), providing further evidence that the primary interaction requires exposure of the underlying β -sheet face of the B30.2 domain (site 2). The L450R mutation did not have the same effect (Figure 2c), possibly because L450 is partly surface exposed, and the Arg replacement could be organized to face outward, allowing binding with some loss of affinity. Notably, F449 also forms a strong hydrophobic interaction with the underlying β -sheet and this may, in part, maintain the interaction when L450 is mutated. In contrast, L446 is completely buried and replacement with Arg in this position is likely to disrupt the interaction with the rest of the protein.

Collectively, these results identify a putative second interaction site (site 2) on the TRIM25 B30.2 domain that is involved in binding to the RIG-I CARDs. Evidence for a second binding site on other TRIM B30.2 domain-containing proteins can also be found in an analysis of disease-causing polymorphisms in the MEFV gene, which encodes Pyrin (TRIM20) [26]. These mutations cause a debilitating autoinflammatory disease called Familial Mediterranean Fever. One of the three most common mutations, V726A, greatly reduces the interaction with caspase-1 [27] and maps to the β -sheet face of the Pyrin B30.2 domain in a region analogous to site 2 in TRIM25 (Figure 2e).

A potential TRIM25-interaction site in CARD2 of RIG-I

The TRIM25 B30.2 domain has been reported to interact with CARD1 of RIG-I [25]. Given that the TRIM25 putative site 2 : CARD interface is hidden by interaction with the TRIM25 α 1-helix (Figure 1b), we investigated whether the RIG-I interaction with site 2 of TRIM25 could be mediated by a similar helix in the RIG-I CARDs. A multiple sequence alignment was performed between the amino acid sequences of human and mouse RIG-I 2CARD and the TRIM25 α 1 helix. Somewhat surprisingly, this revealed a sequence in CARD2 (and not CARD1), which had amino acid sequence similarity to the TRIM25 α 1 helix (corresponding to the fourth α -helix in CARD2; C2 α 4) (Figure 3a).

To explore this further, we tested binding of the TRIM25 B30.2 domain with various RIG-I CARD constructs. HEK293T cells were transfected with GST-tagged human RIG-I CARD1-only, CARD2-only and 2CARD (containing both CARD1 and CARD2) constructs, together with FLAG-tagged α 1-containing or deleted (α 1), human TRIM25 B30.2 domain or SPSB2 SPRY (as a negative control). GST-CARD complexes were recovered using glutathione-Sepharose beads, and the presence of the TRIM25 B30.2 domain was detected by Western blot with anti-FLAG antibody (Figure 3b, upper panel). Human TRIM25 B30.2 α 1 interacted specifically with all three RIG-I CARD constructs but not with the GST-only control, while the α 1-containing B30.2 also bound to the RIG-I CARD constructs (albeit very weakly) (Figure 3b).

To clarify the role of individual residues in RIG-I in mediating an interaction with the TRIM25 B30.2 domain, we resolved a 2.5 Å crystal structure of the mouse RIG-I CARDs (residues 1–189) by molecular replacement, using duck RIG-I CARD domains (PDB ID

4A2Q) as a model. Five molecules were present in the asymmetric unit, with four of these interacting to form a tetramer (Supplementary Table S1 and Supplementary Figure S2a; Figure 3c). The structure is consistent with the published human RIG-I tetramer structure [19]. However, in contrast with the human structure, the mouse tetramer crystallized in the absence of ubiquitin. Structural alignment of the mouse RIG-I 2CARD monomer with the duck and human RIG-I 2CARD monomers demonstrated nearly identical tertiary structure (RMSD: 0.6 Å over 164 residues) and a similar CARD2 conformation (Supplementary Figure S2b,c). The conserved residues in RIG-I C2α4 align structurally with the TRIM25 α1 residues that interact with the underlying site 2 (Figure 3d) and could potentially form additional interactions with site 2 (Supplementary Figure S2f). However, the C2α4 helix is not solvent-exposed and is hidden behind two other α-helices, forming an integral part of the tightly packed hydro-phobic core of CARD2 (Figure 3e). Essentially, an interaction with the TRIM25 site 2 would require unfolding of CARD2 and the displacement of α1 from TRIM25. While this seems unlikely, it is theoretically possible that during RIG-I activation, a major conformational change in CARD2 could release these helices to allow access to the underlying C2α4 helix.

Analysis of the mouse RIG-I 2CARD monomer structure suggested that a hydrogen bond (2.7 Å) between the side chain of S162, above the C2α4 and the main chain of C130 at the apex of the loop connecting the two outer helices, together with non-polar contacts between L131 and C2α4 residues, was responsible for holding the helices in place (Figure 3e). We therefore predicted that mutation of L131 and S162 might encourage release of the helices and promote the interaction with TRIM25. The similarity between the TRIM25 α1 and the RIG-I C2α4 helix further suggested that G151 and L159 in RIG-I might be involved in the interaction with TRIM25. L131, S162, G151 and L159 were therefore mutated to alanine. The RIG-I G151A mutant bound to TRIM25 B30.2 α1 to a similar extent as the wild-type 2CARD, while the L159A mutation resulted in a modest decrease in the interaction. As predicted, mutation of either L131 or S162 increased RIG-I 2CARD binding to TRIM25 (Figure 3f, upper panel).

To test the functional relevance of the RIG-I 2CARD loss-of-binding and gain-of-binding mutations, we utilized an IFN-β reporter assay. In this system, overexpression of the RIG-I CARDS alone in HEK293T cells is sufficient to induce IFNβ production; the pathway is further activated by co-expression of full-length TRIM25. Consistent with the modest reduction in binding to TRIM25 (Figure 3f), mutation of G151 or L159 in the CARD2 Cα4 helix resulted in reduced production of IFN-β in response to TRIM25-mediated activation of the RIG-I CARDS (Figure 3g). The increase in binding observed with the S162A mutant was supported by a corresponding increase in IFN-β promoter activity, while the L131A mutation resulted in a modest reduction in signaling, although in the latter the levels of TRIM25 were consistently reduced (Figure 3g).

In summary, the data identify a new binding site on the TRIM25 B30.2 domain, which mediates an interaction with the RIG-I CARDS. Surprisingly, the interaction can occur with either of the two RIG-I CARD domains.

Small-angle X-ray scattering analysis reveals TRIM25 B30.2 domains on a flexible tether

To determine the relative orientation of the B30.2 domains to the CCD and visualize how the TRIM25 B30.2 domain might recruit the RIG-I CARDs, we performed small-angle X-ray scattering (SAXS) analysis of a TRIM25 CCD–B30.2 dimeric complex. His-tagged mTRIM25(CCD–B30.2)180–634 was expressed in *E. coli* and purified by affinity purification and size-exclusion chromatography. SAXS analysis revealed that the CCD–B30.2 was monodisperse in solution and had a radius of gyration of 62.9 Å (Figure 4a), with a maximal dimension of 215 Å (Figure 4b and Supplementary Table S2). The Porod volume estimate of 196 782 is most consistent with CCD–B30.2 existing as a homodimer in solution with molecular mass of ~100 kDa: a finding confirmed independently by analytical ultracentrifugation (Supplementary Figure S5). We proceeded to model the relative dispositions of the mB30.2 and CCD using BUNCH, where the crystal structures of the mB30.2 and CCD connected by a flexible bead linker were fit as rigid bodies to the experimental SAXS data. Interestingly, multiple models featuring diverse dispositions of the B30.2 domains relative to the CCD were obtained that fit the experimental data equivalently (Figure 4c and Supplementary Figure 4a–c). The diversity of relative domain positions in these rigid body models suggested that the CCD–B30.2 construct was intrinsically flexible and can adopt multiple conformations. This notion was supported by Porod–Debye analysis of the scattering data (Supplementary Figure S4d), where the absence of a plateau in the plot indicated that the CCD–B30.2 construct does not exist as a compact assembly in solution. Collectively, these analyses are most consistent with the idea that the B30.2 domains are surprisingly mobile and support a model whereby in solution, the B30.2 domains move freely on a flexible tether, sampling the surrounding space for target interactors.

Discussion

We have identified a putative second binding site on the TRIM25 B30.2 domain (site 2), consisting of a hydro-phobic patch located on the opposite face of the domain from site 1 and which, in our crystal structure, is hidden beneath the N-terminal α 1 helix (Figure 1b). The α 1-helix is positioned between the TRIM25 CCD and B30.2 domains. Given that the CCD forms an elongated dimer [28], it is possible that in the dimerized protein, the α 1-helix may be contiguous with the CCD and lifted away from the β -sheet face of the B30.2 domain. This concept is further supported by a B-factor analysis of the mouse TRIM25 B30.2 domain structure, which suggests that the N-terminal α -helix is more flexible than the rest of the molecule (Supplementary Figure S1b). FLAG-tagged α 1-helix-containing, but not α 1-helix-deleted, TRIM25 B30.2 domain was able to immunoprecipitate endogenous TRIM25 (Supplementary Figure S1e, data not shown), implying that this interaction may be mediated by α 1-contacts with a distal region of the TRIM25 CCD and is consistent with structural studies, showing an antiparallel arrangement of the TRIM25 CCD dimer [28]. While the CCD crystal structure revealed inter-digitating helical hairpins, it did not include the 50 amino acid region (including the α 1 helix) that precedes the N-terminal end of the B30.2 domain. The exact positioning of the α 1 helix in the context of the TRIM25 dimer is therefore unresolved, but one possibility is that the TRIM25 dimerization interface involves both the α 1 helix and the CCD, with the net result being exposure of the novel TRIM25 B30.2 domain site 2.

The human RIG-I 2CARDs form a tetrameric complex, which is required for signaling and is dependent on ubiquitin-mediated stabilization [19]. Our crystal structure of the mouse RIG-I CARD domains reveals the formation of a similar tetramer; however, in contrast with the human structure, the mouse tetramer formed in the absence of ubiquitin. In both tetramers, each 2CARD monomer adopts an identical conformation to that of the 2CARDs in the auto-repressed conformation of full-length duck RIG-I [12] (Supplementary Figure S2b). Ubiquitin-independent tetramerization is consistent with earlier work, demonstrating that K63-linked ubiquitination was dispensable for RIG-I signaling in the presence of long RNA [29]. RIG-I required K63-linked ubiquitination to induce signal transduction when short RNA was used, suggesting that the long RNA facilitated a 'proximity-induced' mechanism of RIG-I activation [29]. Although our mouse tetramer structure suggests that murine RIG-I may exhibit a greater propensity for ubiquitin-independent tetramer formation, the ubiquitin-independent formation of RIG-I tetramers may also be a product of the high protein concentration in the crystallization conditions and is unlikely to reflect RIG-I signaling *in vivo*.

A model for TRIM25 recruitment of binding partners

Our data reveal a complex interaction between TRIM25 and RIG-I involving two interaction sites on the TRIM25 B30.2 domain, and with apparently unique interaction sites on each of the RIG-I CARDs. These data raise many questions regarding exactly how TRIM25 interacts with RIG-I and how this impacts on RIG-I signaling. For instance, we might have expected that a sequence in CARD1 would match the TRIM25 $\alpha 1$ helix. This was not the case and we are unable to explain how CARD1 interacts with the TRIM25 B30.2 domain. It is possible that more than one sequence motif can interact with site 2 on the TRIM25 B30.2.

Although we did not identify a sequence in CARD1 that matched the TRIM25 $\alpha 1$ helix, surprisingly, we did identify a helix in CARD2 (Ca4) with sequence similarity to the $\alpha 1$ helix. However, Ca4 forms an integral part of the hydrophobic core of CARD2, and exposure of the Ca4 helix for binding to TRIM25 would necessitate a large energy cost and major structural reorganization. The interaction with the isolated CARD2 domain appeared specific, and mutation of L131 and Ser162 enhanced the interaction, as might be predicted if the Ca4 helix was to become accessible for binding. While these results confirmed that the complex between TRIM25 and CARD2 is influenced by these residues, they did not distinguish between destabilization of the complex and destabilization of CARD2 which could enhance a 'sticky' or nonspecific interaction. Despite these caveats, it is tempting to speculate that phosphorylation of S162, together with binding to CARD1, might induce a conformational change that enables TRIM25 binding. We were unable to demonstrate an *in vitro* interaction using the bacterially expressed RIG-I 2CARD and TRIM25 B30.2 domain lacking the $\alpha 1$ helix (residues 455–634) (data not shown), and this may be a further indication that post-translational modification is required for binding.

The structure of the 2CARD monomer does not allow for the two adjacent CARDs to interact with two sites on one TRIM25 B30.2 domain, leaving the possibility that each B30.2 domain could potentially interact with two RIG-I molecules and/or the CARDs could 'bridge' two TRIM25 B30.2 domains. Regardless, the spatial constraints imposed by the

tetramer suggest that dissociation of TRIM25 would be required for subsequent CARD2 interactions with the MAVS CARD and again we need to consider the energy cost if such a model was borne out.

The SAXS and AUC analysis of the TRIM25 CCD–B30.2 construct is consistent with the native TRIM25 existing as a dimer. The SAXS data also indicate a surprising degree of variation in the positioning of the TRIM25 B30.2 domains relative to the CCD (Figure 4), supporting a model whereby the B30.2 is attached to the CCD by a flexible linker region and can move freely in solution. This concept is compatible with each B30.2 domain recruiting two RIG-I proteins or other B30.2-interacting proteins. There are 38 TRIM proteins that contain a RING domain, B-Boxes and a B30.2 domain adjacent to a CCD [30]. The prevalence of this motif suggests that our data can be extrapolated to the wider TRIM family, and we hypothesize that the flexibility is an inherent feature of these proteins, with the CCD acting as a scaffold to enable B30.2 domain capture of its substrates, positioning them for ubiquitination by the RING domains.

The identification of two protein-interaction sites on the TRIM25 B30.2 domain has broader implications for our understanding of how the greater TRIM/B30.2 family functions. For example, the rhesus macaque TRIM5 α residues involved in binding the HIV-1 viral capsid map to a region within the B30.2 domain [31,32], which is analogous to site 1, as do residues involved in SPSB and TRIM21 recognition of inducible nitric oxide synthase and IgG F_c, respectively [33,34]. Yang et al. [32] also discovered various mutations in loops SP1 and SP2 that did not affect binding to the HIV-1 viral capsid, but disrupted retroviral restriction ability, suggesting that the TRIM5 α B30.2 domain also interacts with one or more as yet undiscovered cofactors, and providing further evidence that the SPRY/B30.2 domain may interact with multiple target proteins. Additional 'site 2' interactions are suggested by the V726A mutation located in an analogous region of the Pyrin B30.2 domain [26] (Figure 2e) and a recent gain-of-function mutation located in the Pyrin CCD adjacent to the B30.2 region (T577N) [35], which may increase accessibility to an underlying site 2.

Viral pathogens remain a significant health problem in the 21st century, largely because of the capacity of the viral genome (particularly influenza) to adapt and change rapidly, and the potential for rapid transmission of disease in our highly mobile society. The influenza viral protein NS1 is known to bind to the TRIM25 CCD and inhibit dimerization [17,36], thereby abrogating the TRIM25 interaction with RIG-I and attenuating production of type I IFNs. A deeper biochemical and structural understanding of TRIM25 dimerization, and its interaction with RIG-I and/or other target complexes, could offer other avenues for the development of therapeutics that enhance host anti-viral immunity, to benefit at-risk, immunocompromised individuals who are highly vulnerable to viral infections, such as influenza.

Materials and methods

Plasmid construction

GST-mRIG-I(2CARD)^{1–189} was generated by PCR and subcloned into the bacterial expression vector pGEX-2T. Histidine (His)-mTRIM25(CCD–B30.2)^{180–634} was subcloned

into the bacterial expression vector pACYC. Constructs encoding proteins with a C-terminal FLAG or MYC tag were subcloned into pEF-Flag-I, a derivative of pEF-BOS [37]. Point mutants in the mouse TRIM25 B30.2 domain (residues 440–634 or $\alpha 1$ construct residues 455–634), and the mouse RIG-I 2CARDs (residues 1–200), were generated using the PCR technique, splicing by overlap extension. The mammalian expression construct for GST-mRIG-I(2CARD) has been described previously [17]. The construct encoding an SPSB2 (SSB-2) protein with an N-terminal FLAG (FLAG-SPSB2) has been described previously [38].

Protein production

Recombinant glutathione-S transferase (GST)-fusion RIG-I CARDs (residues 1–189) and His-tagged TRIM25 CCD–B30.2 (residues 180–634) were expressed in *E. coli* BL21 (DE3) cells and purified as described in Supplementary Information.

Crystallization

Purified RIG-I^{1–189} protein was concentrated to 10 mg/ml in 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid], pH 6.0, containing 75 mM NaCl and 2 mM DTT. Crystallization screens using the sitting-drop/vapor diffusion method at 281 K were performed at the Bio21 Collaborative Crystallization Centre (C3), Parkville, Australia. The best diffracting crystals grew from a reservoir solution of 0.2 M ammonium sulfate and 26% (w/v) polyethylene glycol 3350/0.1 M Bis–Tris (pH 6.5).

Data collection and structure determination

Crystals were transferred to a cryoprotectant solution consisting of a reservoir solution supplemented with 20% ethylene glycol, then mounted in a nylon loop and flash-frozen in liquid nitrogen. Diffraction data were collected on beamline MX2 at the Australian Synchrotron in Melbourne (<http://www.synchrotron.org.au/>). Data were integrated using XDS and scaled using XSCALE [39] from the CCP4 suite [40]. A molecular replacement solution was found using PHASER [41] and the two individual CARD domains from duck RIG-I (PDB ID code 4A2Q) as independent search models. Iterative rounds of model building and refinement were performed in COOT [42] and PHENIX [43], respectively. Figures were generated using Pymol (Schrödinger). Co-ordinates and structure factors of murine RIG-I CARD domains (1–189) have been deposited in the Protein Data Bank under ID code 6BZH. CC1/2 was used as a guide to select an appropriate cutoff for the X-ray data, as explained in Karplus and Diederichs [44].

Co-immunoprecipitation and affinity purification

HEK293T cells were maintained in DMEM supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% fetal bovine serum, and transiently transfected using EugeneHD or Eugene6 (Promega) according to the manufacturer's instructions. After 48h of transfection, cells were lysed in NP-40 buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% [v/v] NP-40 and protease inhibitor cocktail [Roche Applied Bioscience]). Post-centrifuged lysates were incubated with anti-FLAG M2-agarose beads (Sigma–Aldrich) for 3–4 h at

4°C, or alternatively, GST-fusion proteins were recovered with glutathione-Sepharose beads (GE Healthcare), prior to Western blotting as described in Supplementary Information.

Luciferase reporter assay

HEK293T cells were transfected with 500 ng of an IFN- β -responsive promoter–Firefly luciferase reporter gene, 20 ng of a Renilla luciferase reporter (HSV-TK; Promega) and constructs encoding MYC-tagged RIG-I 2CARD and FLAG-tagged full-length mTRIM25 proteins. Luciferase activity was quantified using the Luciferase Assay Dual-Reporter Kit (Promega) and an automated LUMIstar Galaxy plate reader (BMG Technologies).

SAXS data collection and analyses

SAXS data were collected at the Australian Synchrotron [45] using the inline size-exclusion chromatography setup, as described in refs [46,47]. The ATSAS suite of software was used for all subsequent SAXS data analyses [48]. Rigid body modeling was performed using *BUNCH* [49], using a starting model comprising the two known domain structures, mouse TRIM25 B30.2 (PDB: 4B8E) and human TRIM25 coiled-coil domain (PDB: 4LTB), with regions absent from the component domain structures modeled as flexible dummy atoms by *BUNCH*, and P2 symmetry imposed with distance restraints between protomers in the coiled-coil domain homodimeric crystal structure.

Additional experimental procedures are available in Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AUC	analytical ultracentrifugation
CARD	caspase activation and recruitment domain
CCD	coiled-coil domain
CTD	C-terminal domain
GST	glutathione-S transferase

IFN	interferon
IRF	interferon-regulatory factor
MAVS	<u>mitochondrial anti-viral signaling</u>
MDA5	melanoma differentiation-associated gene 5
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
RIG-I	<u>retinoic acid-inducible gene-I</u>
RLRs	retinoic acid-inducible gene-I (RIG-I)-like receptors
SAXS	small-angle X-ray scattering
TRIM25	<u>tripartite motif 25</u>

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Figure 1. Domain architecture of RIG-I and TRIM25 and structure of the TRIM25 B30.2 domain.

(a) Schematic showing the domain architecture of RIG-I (top) and TRIM25 (bottom). (b) Cartoon representation of the 3D structure of the TRIM25 B30.2 domain (red) with the $\alpha 1$ helix in yellow (PDB: 4B8E). Side chains of key residues in site 1 (gray), site 2 (green) and the $\alpha 1$ helix (L446, L450; yellow) are highlighted.

Figure 2. Deletion of the TRIM25 $\alpha 1$ helix reveals a novel RIG-I CARD-binding site. (a) HEK293T cells were transfected with human (h) and mouse (m) FLAG-tagged TRIM25 B30.2 constructs which either contained (B30.2) or lacked (B30.2 $\alpha 1$) the $\alpha 1$ helix, or a FLAG-tagged SPSB2 construct, together with either h or mRIG-I 2CARD constructs. Cells were lysed and anti-FLAG immunoprecipitates were analyzed for the presence of RIG-I 2CARD by Western blotting with anti-MYC antibodies (upper panels). The blots were stripped and reprobed with anti-FLAG antibodies (upper middle panel). Cell lysates were blotted to show the levels of MYC- and FLAG-tagged constructs (lower middle and bottom panels, respectively). (b) HEK293T cells were transfected with a series of wild-type (WT) and mutant FLAG-tagged mB30.2 $\alpha 1$ constructs together with the MYC-tagged mRIG-I 2CARD construct and analyzed as in (a). Note that these data show the effects of mutating key residues in the B30.2 domain and binding of MYC-tagged 2CARD is a negative result with no loss of function. (c) HEK293T cells were transfected with WT or mutant (L446R, L450R) mB30.2 constructs together with the MYC-tagged mRIG-I 2CARD construct and

analyzed as in (a). In contrast with (a), these data show the effects of mutating residues within the $\alpha 1$ helix and in this instance, the binding of MYC-tagged 2CARD reflects a gain of function. (d) HEK293T cells were transfected with constructs expressing, either FLAG-tagged m or hRIG-I 2CARD, mSPSB2 SPRY domain, hMAVS or hMAVS CARD, together with MYC-tagged mTRIM25 B30.2 $\alpha 1$. Anti-FLAG immunoprecipitates were analyzed for the presence of mB30.2 $\alpha 1$ by Western blotting with anti-MYC antibodies (upper panels). The blots were reprobed with anti-FLAG antibodies (upper middle panel). Cell lysates were blotted to show the levels of MYC and FLAG-tagged constructs (lower middle and bottom panels, respectively). (e) The three most common Familial Mediterranean Fever-associated polymorphisms in the B30.2-encoding sequence of the MEFV gene are shown in yellow mapped onto a cartoon representation of the Pyrin B30.2 domain 3D crystal structure (purple) (PDB ID: 2WL1) (Weinert et al. [26]).

Figure 3. Identification of a potential novel interaction interface in RIG-I CARD2.

(a) Alignment of the TRIM25 $\alpha 1$ helix with the RIG-I CARD sequences revealed sequence homology with an α -helix located in RIG-I CARD2 (residues 140–180, C2 $\alpha 4$). Sequences were extracted from the hTRIM25 and mRIG-I sequences (NCBI GenBank database). Multiple sequence alignment was conducted in ClustalX 2.0.12. Bar graphs represent a matrix score for sequence substitution. *, amino acid identity and :, similarity. The positions of L446 and L450 are indicated by red arrows. (b) HEK293T cells were transfected with the FLAG-tagged hTRIM25 B30.2, B30.2 $\alpha 1$ or mSPSB2 SPRY domain, in the presence of GST-RIG-I single or 2CARD constructs. RIG-I CARD complexes were recovered with glutathione-Sepharose beads, and the presence of TRIM25 B30.2, B30.2 $\alpha 1$ or SPSB2 was detected by Western blotting with anti-FLAG antibodies (upper panel). Membranes were reprobed with anti-GST antibodies (upper middle panels), and the expression of all proteins was confirmed by Western blotting of cell lysates (lower panels). (c) Cartoon representation of the 2.5 Å crystal structure of the mouse RIG-I 2CARD tetramer, with each molecule colored differently. (d) Cartoon showing part of the mouse TRIM25 B30.2 domain structure (PDB ID: 4B8E). Conserved residues in the RIG-I C2 $\alpha 4$ helix are highlighted in dark green on the mouse TRIM25 $\alpha 1$ helix (yellow). (e) Cartoon of the crystal structure of mouse RIG-I CARD domains as a monomer. CARD1 (blue-white) and CARD2 (cyan) are separated by a short linker (teal). The conserved C2 $\alpha 4$ helix is shown in green and the outside helices in wheat. Inset: L131 and S162 (pink) form polar and non-polar contacts (dashed lines) that hold the outer helices in place. (f) HEK293T cells were transfected with FLAG-tagged hTRIM25 B30.2 $\alpha 1$, in the presence of MYC-tagged RIG-I 2CARD WT or mutant constructs. TRIM25 B30.2 $\alpha 1$ complexes were recovered by immunoprecipitation (IP) with anti-FLAG antibodies and the interaction with RIG-I 2CARD was detected by Western blotting with anti-MYC antibodies (upper panel). Protein expression was confirmed in cell lysates by Western blotting (middle and bottom panels). (g) HEK293T cells were transfected with IFN- β -responsive-Firefly reporter and HSV-TK-Renilla luciferase constructs, together with MYC-tagged RIG-I 2CARD WT and mutant constructs and full-length FLAG-tagged TRIM25. Cells were lysed and Firefly luciferase activity was normalized to Renilla activity. Data are expressed as the mean of triplicates $\pm$ SD. Protein expression was analyzed in cell lysates by Western blotting.

Figure 4. SAXS analysis reveals B30.2 domains on a flexible tether.

(a) Scattering intensity profile for TRIM25 CCD–B30.2 (residues 179–634) with inset Guinier plot showing data in the q range. Linearity of the Guinier plot indicates that neither high-molecular-mass aggregates nor interparticle interference contributes measurably to scattering. (b) Fourier transformation of the scattering intensity yields the pair-wise interatomic distance distribution function $P(r)$, calculated using *GNOM*. The maximum particle dimension, D_{max} , is 215 $\text{\AA}$. (c) Rigid body modeling to fit the experimental scattering data was performed using *BUNCH* and the crystal structures of mouse TRIM25

B30.2 (PDB: 4B8E) and human TRIM25 CCD (PDB: 4LTB), with regions absent from the component domain structures modeled as flexible dummy atoms. The compositions shown had a χ value of <0.38.

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