

Original Paper

RAIDD Mediates TLR3 and IRF7 Driven Type I Interferon Production

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Key Words

IRF7 • Innate immunity • TLR • RAIDD

Abstract

Background/Aims: Viral infections represent a global health problem with the need for new viral therapies and better understanding of the immune response during infection. The most immediate and potent anti-viral defense mechanism is the production of type I interferon (IFN-I) which are activated rapidly following recognition of viral infection by host pathogen recognition receptors (PRR). The mechanisms of innate cellular signaling downstream of PRR activation remain to be fully understood. In the present study, we demonstrate that CASP2 and RIPK1 domain-containing adaptor with death domain (CRADD/RAIDD) is a critical component in type I IFN production. **Methods:** The role of RAIDD during IFN-I production was investigated using western blot, shRNA mediated lentiviral knockdown, immunoprecipitation and IFN-I driven dual luciferase assay. **Results:** Immunoprecipitation analysis revealed the molecular interaction of RAIDD with interferon regulatory factor 7 (IRF7) and its phosphorylating kinase IKK ϵ . Using an IFN-4 α driven dual luciferase analysis in RAIDD deficient cells, type I IFN activation by IKK ϵ and IRF7 was dramatically reduced. Furthermore, deletion of either the caspase recruitment domain (CARD) or death domain (DD) of RAIDD inhibited IKK ϵ and IRF7 mediated interferon-4 α activation. **Conclusion:** We have identified that the adaptor molecule RAIDD coordinates IKK ϵ and IRF7 interaction to ensure efficient expression of type I interferon.

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Introduction

Innate immune activation is critical in inducing anti-viral immunity through cytokine production and adaptive immune priming [1]. Antigen presenting cells including dendritic cells (DCs) recognize pathogens through pathogen recognition receptors (PRRs) [1]. Specifically, DCs sense viral nucleic acids via PRRs including membrane bound toll like receptors TLRs, which results in production of type I interferon (IFN-I) and pro-inflammatory cytokines [1]. TLR9 senses DNA such as CpG oligoribonucleotides [2], while TLR7 recognizes single stranded RNA (mimicked by imiquimod) [3, 4] and TLR3 recognizes double stranded RNA (mimicked by PolyI:C) [5]. Following ligand binding, the intracellular domains of TLR7 and TLR9 bind a multi-protein complex composed of MyD88, TRAF6 [6], and the IL1-receptor interacting kinase (IRAK) family members IRAK4 and IRAK1, which can also be activated by other membrane proteins such as TREML4 [7, 8]. The activated complex triggers activation of IKK ϵ , TBK1, IKK α and/or IRAK1. The later of which phosphorylate the transcription factor interferon regulatory factor 7 (IRF7) [9-11]. In contrast to TLR7 and TLR9, TLR3 signals via the adaptor molecule TRIF [12]. TRAF3 interacts with TRIF and is critical for the activation of downstream kinases IKK ϵ and TBK1 [13, 14]. Coordinated activation of TLRs results in the phosphorylation of the transcription factors IRF7 and IRF3 [15-17]. Consequently, phosphorylated IRF7 and IRF3 then translocate into the nucleus followed by specific gene promoter binding to induce IFN-I gene transcription [18-20].

The death domain superfamily proteins are involved in various intracellular signaling cascades to orchestrate the innate and adaptive response against infection [21] (Table 1). In this study we have characterized one of the death domain superfamily proteins, the RIP associated ICH-1/CED-3-homologous protein with a death domain (RAIDD) carrying two functional domains, a death domain (DD), as well as a caspase activation and recruitment domain (CARD) [22]. RAIDD is known to interact with RIP1 and is capable of inducing apoptosis [23]. Specifically, RAIDD plays a role in PIDD and heat shock induced apoptosis by recruiting caspase-2 [24, 25], although caspase-2 can also be activated independently of both PIDD and RAIDD [26]. Additionally, recent findings suggest that RAIDD suppresses inflammatory signaling mediated by NF- κ B signaling in T cells by sequestering BCL10 [27]. However, the involvement of RAIDD during PRR signaling and its consequent innate immune response has not been described.

In this study we found that TLR3 driven IFN-I activation is inhibited in RAIDD deficient cells. Mechanistically, RAIDD interacted with IRF7 and its phosphorylating kinase IKK ϵ , thereby stimulating IKK ϵ mediated IRF7 phosphorylation and promoting IFN-I production. Overall, these studies identify a novel adaptor molecule RAIDD, with an essential role in TLR3 driven IFN-I production.

Materials and Methods

Cell culture, Cloning, Plasmid preparation and Transfection

Human RAIDD was amplified using 5'-TGC GGC CGC_GGA GGC CAG AGA CAA ACA AGT-3' and 5'-TGT CGA CGA GGC ACC ATC ACT CCA ACA-3'. Similarly, human RAIDD CARD domain with 279 bp in length was deleted (Δ C) using 5'-TAG CGG CCG CGA CCG ACC TGC CTG CAG GT-3' and 5'-TAG AGT CGA CGA GGC ACC ATC ACT-3'. The 249bp of human RAIDD DD domain were deleted (Δ D) using 5'-ATG ACA AGC TTG CGG CCG CGG A-3' and 5'-AGA GTC GAC_TCA TGA TGG GGA GCT GTT GA-3'. Amplified products were initially cloned into the pGEM-T-easy cloning vector, thereafter subcloned into the p3XFlag-CMV-7.1 vector using *NotI* and *SalI* restriction enzymes. Human IKK ϵ was amplified using 5'-AAG CTG GCT AGC GTA TGC AGA GCA CAG CCA ATT A-3' and 5'-GAG ACT AGG GTC AGC GAC ATC AGG AGG TGC TGG-3' and cloned into pGEM-T-easy cloning vector, thereafter subcloned into pcDNA3.1/zeo with C-terminal HA-tag using *NheI* and *PshAI* restriction enzymes. Murine nucleotide sequence encoding RAIDD-C-term-HA was custom-synthesized by Gene script and cloned into pcDNA3.1/zeo (based on full ORF of NM_009950.2 with codon optimization for mouse expression). pBEN2-eYFP-IRF7A was obtained from CK. pUNO1-hIKK ϵ was purchased from Invivo-

Table 1. Impact of Death Domain superfamily proteins on signaling cascades. The death domain superfamily proteins described to be involved in various intracellular signaling cascades (adopted from Park et al. [21])

Signal Cascade	Domains	Protein Components
TLR Complex	DD	Myd88, TIRAP, IRAKs, TRAF6
Inflammasome	PYD	NALP1, ASC, Caspase-1, Caspase-5
DISC	DD,DED	Fas, FADD, Caspase-8 or 10
PIDDosome	DD, CARD	PIDD, RAIDD, Caspase-2
Apoptosome	CARD	Apaf-1, Cytochrome C, ATP/dATP, Caspase-9
TCR/BCR Signaling	CARD	CARMA1, BCL10, MALT1, RAIDD
NOD Pathways	CARD	Nod1 or Nod2, RIP2, Caspase-1
RIG Pathways	CARD	RIG1 or MDA5, MAVS

gen (puno1-hikkε). All the expression plasmids were amplified using promega maxi pre kit according to the manufactures instructions. Expression plasmids were transfected using lipofectamine 2000 or lipofectamine LTX (Invitrogen) in 293t cells or HEK TLR3 cell lines. 24 h post transfection, cells were processed for further analysis. Unless otherwise indicated, cells were cultured in DMEM containing 10% FCS, L-Glutamine, Penicillin, and Streptomycin.

Stable Knockdown of RAIDD

Lentiviral particles were generated by calcium phosphate transfection of sub-confluent (50-60%) 293TV cells with 10 μ g of shRNA (Origene), 5 μ g each of pMDG1.vsvg, pRSV-Rev and pMDLg/pRRE constructs. Lentiviral particles were collected 24 and 48 hours later, filtered through a 0.45 μ m filter and stored at -80°C. Parental cells (293t cells or HEK TLR3 cells) were infected with lentiviral particles containing the indicated shRNAs and cells were selected with puromycin (48 h, 5 μ g/ml of puromycin). Two different specific RAIDD shRNA's were used in the experiments.

IFN promoter assay

IFN β promoter assay [28] and IFN4 α promoter assays were performed as previously described [29]. Briefly, IFN luciferase promoter and reporters were used in the ratio of 10:1 and with plasmid alone or together with a combination of expression vectors at indicated concentration. The 293t cell lines or HEK TLR3 cell lines (Invivogen), reporter gene activities were measured by dual luciferase reporter assay system (Promega) after 24 h Post-transfection.

Immunoblotting and Immunoprecipitation

Briefly, cells were lysed in PBS containing 1% TX-100 (Sigma), EDTA-free protease inhibitor cocktail (Sigma), Phospho stop (1 tablet/10 mL). Immunoblots were probed with primary anti-RAIDD (abcam ab8426), anti-IRF7 (Cell Signaling 4920 or 13014), and β -actin (Cell Signaling 5125). The pull-down was performed using MultiMACS HA/ GFP Isolation Kit or mAbs recognizing the FLAG.

Statistical analyses

Data are expressed as mean \pm S.E.M. Statistical significant differences between two different groups were analyzed using students t test. Statistical differences between several groups were tested using one-way ANOVA with additional Bonferroni or Dunnett's post-tests. Statistically significant differences between groups in experiments involving more than one analysis time point were calculated using two-way ANOVA (repeated measurements).

Results

RAIDD is critical for IFN-I production after TLR3 stimulation

IFN-I is induced by activation of PRRs including Toll like receptors (TLRs) [30]. Particularly, TLR3 can induce production of IFN-I following stimulation with its agonist PolyI:C [5]. To analyze the impact of RAIDD during TLR3 mediated IFN-I production, we generated HEK TLR3 transgenic cells, which stably express scramble (scrRNA) or shRNA against RAIDD (Fig. 1A). When we challenged these cells with Poly I:C, they showed uniform expression of RAIDD at various time points (Fig. 1A). In order to investigate the presence or absence of TLR3 medi-

Fig. 1. RAIDD is critical for IFN-I production after TLR3 agonist treatment. (A) Whole cell lysates were prepared from HEK TLR3 cells expressing scramble RNA and shRNA RAIDD at indicated time points and probed for RAIDD and β actin. (One representative of $n = 3$ is shown) (B) HEK TLR3 cell lines, stably transduced with scramble or RAIDD-targeting shRNA, were transfected with IFN4 α luciferase promoter and Renilla constructs (10:1), and IFN4 α promoter activity was measured after 6h of PolyI:C (25 μ g/ml) treatment. Luciferase activity as normalized to Renilla luciferase activity was shown ($n = 6$ is shown) (C) HEK TLR3 cytosolic and nuclear fractions were isolated at indicated time points and probed for IRF7, HISTONE H3, and GAPDH (one representative of $n = 3$ is shown).

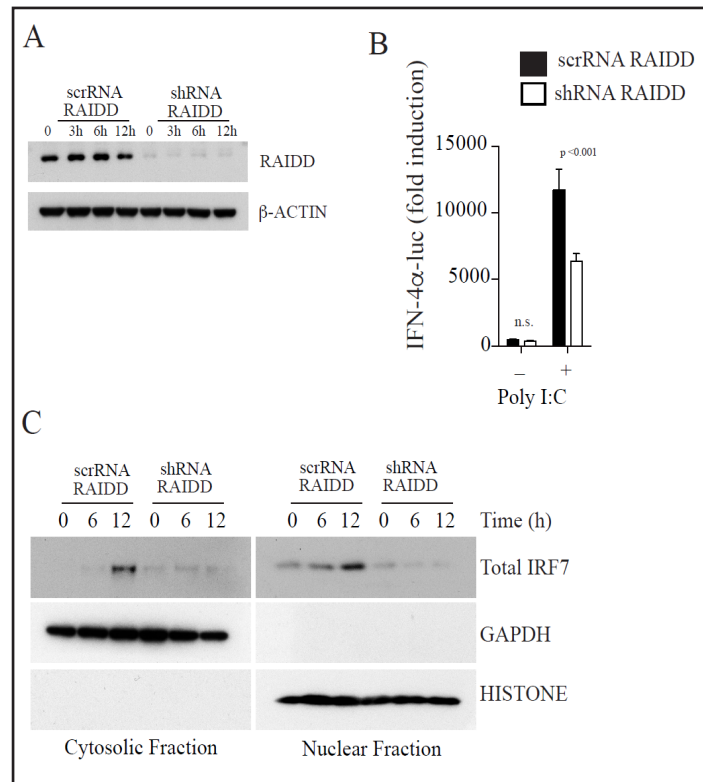
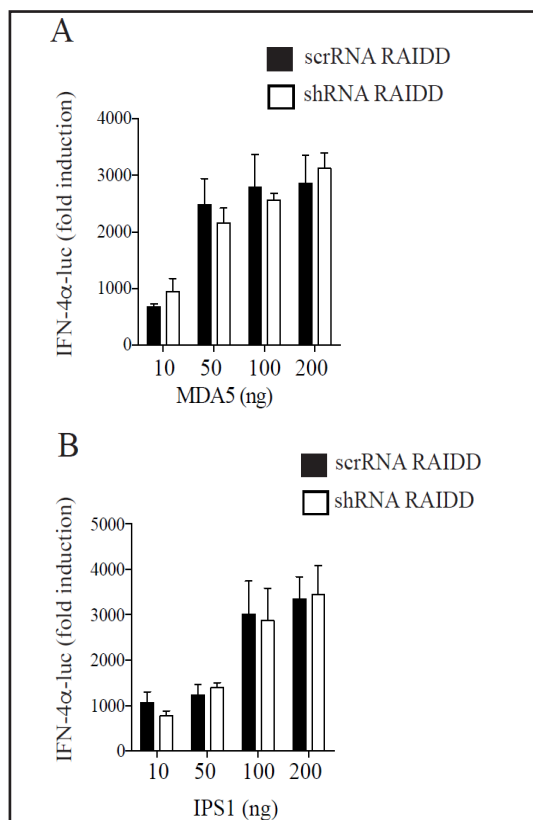


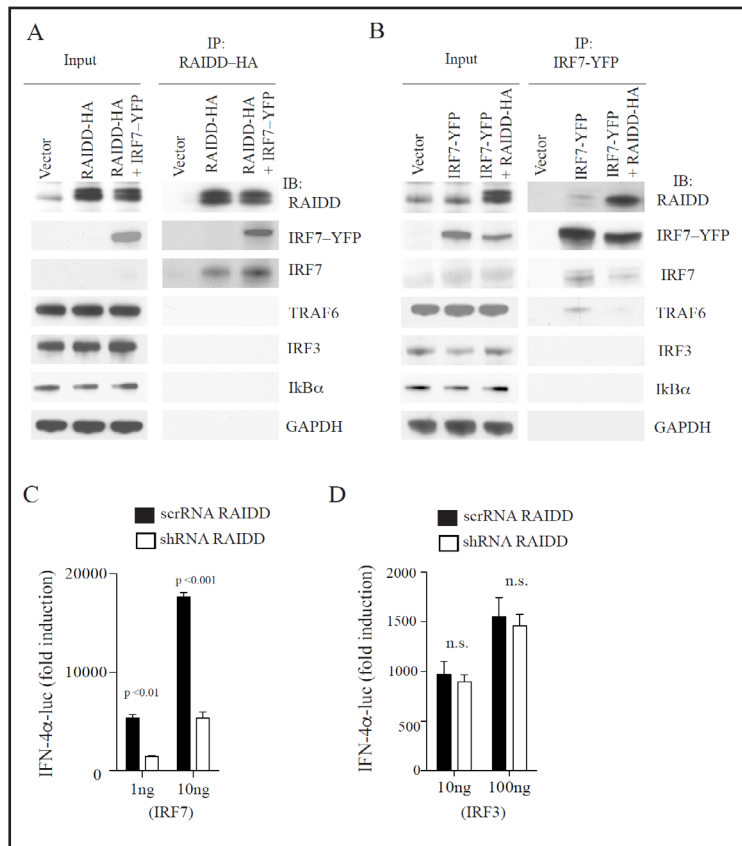
Fig. 2. RAIDD does not affect the RLR ligand MDA5 or its adaptor IPS1 induced IFN-I activation. (A, B) IFN4 α promoter luciferase activities were measured after transient transfection of indicated concentration of IPS1 ($n = 6$ is shown) and MDA5 ($n = 6$ is shown) (24 h post-transfection) from 293t cells stably expressing the scramble and shRNA of RAIDD.

ated IFN-I activation signals, we used IFN4 α promoter driven luciferase. When compared to untreated cells, Poly I:C treatment showed IFN-I activation evident by the expression of luciferase. However, cells lacking RAIDD expression showed significantly reduced activation of IFN-I after Poly I:C treatment (Fig. 1B). Next, we examined the Poly I:C driven nuclear translocation of IRF7, which is one molecular hallmark during IFN-I activation [19, 31, 32]. RAIDD deficient cells showed impaired expression and nuclear translocation of IRF7, when compared to scramble control cells (Fig. 1C). Next, we checked the impact of RAIDD on other dsRNA sentinel receptors such as (MDA5) [33] and its potent adaptor molecule (IPS1) [34]; no notable difference in the activation of IFN-I was observed,



either in presence or absence of RAIDD expression (Fig. 2A and B). Taken together, these data suggest that RAIDD specifically affects the TLR 3 mediated IFN-I activation.

Fig. 3. RAIDD interacts with IRF7 and synergizes with IRF7 in IFN-I activation. (A, B) 293t cells were transfected with RAIDD-HA, IRF7-YFP, or EGFP control plasmids. Whole cell lysates were blotted directly after immunoprecipitation for RAIDD-HA using an anti-HA antibody (A), or immunoprecipitation for IRF7-YFP using an anti-YFP antibody (B). Blotting was performed using anti-RAIDD, anti-IRF7, anti-TRAF6, anti-IRF3, anti-I κ B α or GAPDH antibodies as indicated (one representative of n=3 experiments shown) (Error bars indicate SEM). (C, D) 293t cells stably expressing scrRNA control or shRNA RAIDD were transiently transfected with IRF7 (One representative of n = 3 is shown) (C) or IRF3 (n = 6 is shown) (D) together with IFN4 α luciferase promoter and Renilla plasmids. Dual luciferase activity was measured at 24 h post transfection.



RAIDD triggers IRF7 mediated IFN-I transcription

Next, we investigated whether RAIDD may interact with IFN-I transcription factors. Co-immunoprecipitated RAIDD-HA showed interaction with endogenous IRF7; however, no interaction with the closely related IRF3 could be detected (Fig. 3A). These results were validated by reciprocal co-immunoprecipitation using IRF7-YFP to pull down endogenous RAIDD from the precipitated lysates (Fig. 3B). This interaction could be enriched when both molecules were co-expressed (Fig. 3A and B). Further, RAIDD-HA did not interact with TRAF6 (Fig. 3A) but in sharp contrast to enriched IRF7, which showed interaction as expected (Fig. 3B). The immunoprecipitation of RAIDD-HA or IRF7-YFP showed no sign of interaction with I κ B α (Fig. 3A and B). These results indicate that RAIDD affects IFN-I activation, likely through a mechanism involving a direct molecular interaction with IRF7. Next, we tested the physiological impact of IRF7 and RAIDD association. Hence, we expressed IRF7 in the RAIDD deficient cells and examined IFN-I activation through luciferase expression driven by IFN4 α promoters. Consistent with the molecular interaction, IRF7 mediated IFN-I activation was significantly reduced in the RAIDD deficient cells when compared to scrRNA control (Fig. 3C). Notably, IRF7 mediated IFN production is diminished in RAIDD deficient cells but not completely abrogated (Fig. 3C), indicating a RAIDD independent activation of IRF7. Moreover, RAIDD was dispensable for IRF3 mediated IFN-I activation (Fig. 3D). Together, these data suggest that RAIDD interacts with IRF7 to promote IFN-I transcription.

RAIDD triggers IKK ϵ but not TBK1 mediated IFN-4 α activation

We further investigated the mechanism by which RAIDD affects IRF7 activation. Due to defects noticed in nuclear translocation of IRF7 in RAIDD deficient cells (Fig. 1C) we hypothesized that RAIDD may connect IRF7 to its phosphorylating kinases TBK1 or IKK ϵ [16]. Accordingly, we expressed TBK1 or IKK ϵ in RAIDD competent cells. Strikingly, when compared to scramble controls, we found reduced IKK ϵ driven IFN-I promoter activation in cells with RAIDD deficiency (Fig. 4A), whereas this was not the case for TBK1 (Fig. 4B). The increasing

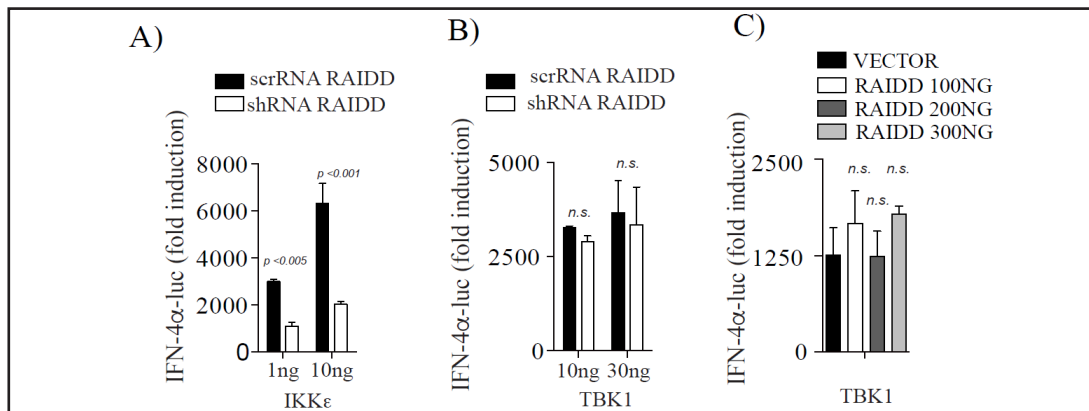
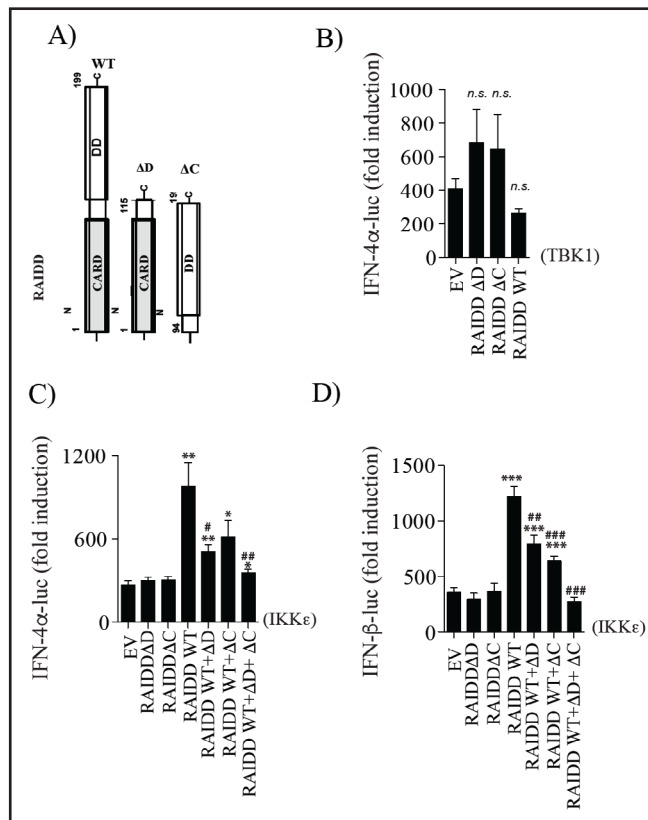


Fig. 4. RAIDD triggers IKK ϵ mediated IFN4 α activation but not the TBK1. (A, B) 293t cells stably expressing scrRNA or shRNA of RAIDD were transiently transfected with IKK ϵ (10 ng/ul) or TBK1 (30 ng/ul) together with the indicated luciferase promoter and measured for IFN4 α luciferase activity 24 h post transfection. (A) (One representative of n = 3 is shown) and (B) (n = 6 is shown). (C) 293t cells were transiently transfected with increasing concentration of RAIDD together with same molecules of TBK1 (30 ng/ μ l). IFN4 α luciferase activities were measured at 24 h post transfection (n = 6 is shown).

Fig. 5. Full length RAIDD enhances IKK ϵ mediated IFN-I activation. (A) A diagram illustrating the constructs of RAIDD, Death domain truncated RAIDD (Δ D), and CARD Domain truncated RAIDD (Δ C) is shown. (B-D) 293t cells were transfected with Empty Vector (EV), RAIDD (Δ C), RAIDD (Δ D), and Full length RAIDD along with TBK1 (n = 6 is shown) (B) and IKK ϵ (n = 6 is shown) (C, D). At 24h after transfection, IFN4 α (B,X) and IFN β (D) luciferase activities were measured.

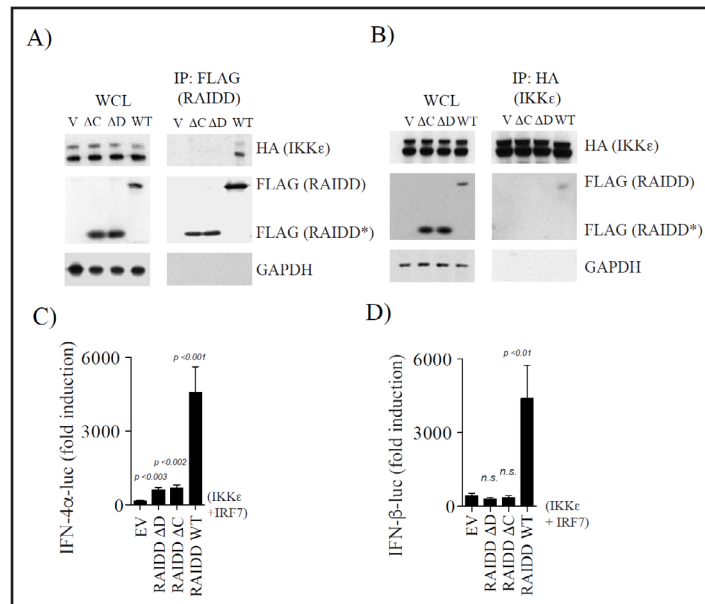


concentration of RAIDD with TBK1 also failed to enhance the basal induction of IFN-4 α luciferase (Fig. 4C). These data indicate, that IKK ϵ but not TBK1 is able to mediate transcriptional activation of IFN4 α and that IKK ϵ requires RAIDD to mediate its effects.

Full-Length RAIDD drives activation of IFN-I mediated by IKK ϵ and IRF7

To investigate which domains of RAIDD trigger IKK ϵ mediated IFN-I activation, we generated deletion constructs of RAIDD, lacking the DD or the CARD (Fig. 5A). We observed

Fig. 6. Full length RAIDD interacts with RAIDD and drives the IFN-I mediated by IKK ϵ and IRF7. (A) 293t cells were lysed followed by immunoprecipitation (IP) using anti-FLAG (RAIDD) (A) or anti-HA (IKK ϵ) (B) antibodies. Immunoblots were probed using anti-FLAG or anti-HA antibodies, along with anti-GAPDH as control (one representative of 3 experiments is shown). (C, D) 293t cells were transfected with Empty Vector (EV), RAIDD (Δ C), RAIDD (Δ D), and Full length RAIDD along with IKK ϵ and IRF7. After 24h, cells were lysed and analyzed for IFN4 α (X) (n=6 is shown) and IFN β (D) (n = 6 is shown) luciferase activities.



that full-length RAIDD significantly increased IFN-I activation, while neither the CARD nor the DD alone promoted IFN-I transcription (Fig. 5B and C). Notably, co-expression of either the DD or the CARD reduced the effects of full-length RAIDD in this setting, indicating that both domains are indeed needed to exert its effects on IFN-I activation (Fig. 5B and C). In support of the previous finding, the different forms of RAIDD have no significant impact on TBK1 mediated IFN-I activation (Fig. 5D).

To further investigate the molecular interaction of RAIDD, we co-expressed IKK ϵ together with empty vectors or CARD or DD or full-length RAIDD and examined for the protein-protein interaction. Although the various mutants of RAIDD were uniformly expressed, only the full-length RAIDD showed interaction with IKK ϵ (Fig. 6A). Consistently, the reciprocal immunoprecipitation also showed interaction of IKK ϵ only with full length RAIDD but not with the mutants (Fig. 6B). Next, we examined the synergetic effects of these components and co-expressed empty vectors or CARD or DD or full length RAIDD together with IKK ϵ + IRF7 following analysis of luciferase activity driven by IFN4 α and IFN β promoters. We observed significant IFN-I activation only in the presence of the full length RAIDD in sharp contrast to both deletion mutants (Fig. 6C and D). We therefore hypothesized that intact RAIDD triggered IKK ϵ and IRF7 mediated induction of IFN-I. Taken together, our data shows that RAIDD interacts with IRF7 and IKK ϵ to coordinate IKK ϵ mediated IRF7 activation and IFN-I production.

Discussion

In this study we have identified RAIDD as a critical regulator of TLR3 driven IFN-I promoter activation, by mediating the interaction between IKK ϵ and IRF7. In cells deficient for RAIDD, IFN4 α and IFN β activation was reduced following immune stimulation. RAIDD consist of two functional domains, the RAIDD-DD domain is reported to be essential for the PIDD interaction [24, 35] and the RAIDD-CARD domain is essential for the binding with caspase 2 [25, 36]. However, later findings argue against this type of interaction [37, 38]. Our data identifies that intact RAIDD is important for PRR driven IFN-I activation.

Pathogens and commensal driven PRR activation results in gradual translocation of transcription factors, such as IRF3, IRF7 and NF- κ B into the nucleus [39]. Deficiency of IRF7 in hematopoietic cells results in limited IFN-I production and fatal viral infection [18, 40]. Consistently, mutations in human IRF7 coincide with severe influenza infection and limited

IFN-I expression [41]. The rheostats of IFN-I effects during viral infection remain to be fully elucidated. While IFN-I is important for limiting viral replication [42], a growing number of other effects have also been identified [31, 32, 39].

IRF7 phosphorylation is regulated by the recently identified factor Pellino3 through the interaction with TRAF6 [32]. However, since we did not observe binding of RAIDD to TRAF6, we speculate that RAIDD triggers activation and nuclear translocation of IRF7 through a Pellino3 independent mechanism. Moreover, the 2'-5'-oligoadenylate synthetase (OAS) family member OASL1 has been shown to prevent IRF7 mRNA translation and consequently IFN-I production [31]. Although we also observed reduced IRF7 expression after stimulation of cells expressing shRNAs targeting RAIDD, the molecular interaction as well as the impaired nuclear translocation of IRF7 in shRNA RAIDD expressing cells suggests an interaction of RAIDD and IRF7 at the protein level. Notably, the transcription factor ELF4 can enhance IRF3 and IRF7 binding following phosphorylation by TBK1 [43]. However, we did not observe a difference in TBK1 mediated IFN-4 α promoter activity following knockdown of RAIDD. Our data identifies an alternative mechanism in which RAIDD connects IRF7 to the upstream kinase IKK ϵ , thereby increasing IFN-I activity.

In conclusion, we identified RAIDD as a crucial adaptor protein for innate immune activation. We have delineated an unexpected TBK1-independent signaling pathway that feeds via IKK ϵ to IRF7 activation. Consequently our data shows that this pathway is crucial for IFN-I production.

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