The Role of Interleukin 1 Receptor-associated Kinase-4 (IRAK-4) Kinase Activity in IRAK-4-mediated Signaling*

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Interleukin 1 receptor (IL-1R)-associated kinase-4 (IRAK-4) is required for various responses induced by IL-1R and Toll-like receptor signals. However, the molecular mechanism of IRAK-4 signaling and the role of its kinase activity have remained elusive. In this report, we demonstrate that IRAK-4 is recruited to the IL-1R complex upon IL-1 stimulation and is required for the recruitment of IRAK-1 and its subsequent activation/degradation. By reconstituting IRAK-4-deficient cells with wild type or kinase-inactive IRAK-4, we show that the kinase activity of IRAK-4 is required for the optimal transduction of IL-1-induced signals, including the activation of JNK, and the maximal induction of inflammatory cytokines. Interestingly, we also discover that the IRAK-4 kinase-inactive mutant is still capable of mediating some signals. These results suggest that IRAK-4 is an integral part of the IL-1R signaling cascade and is capable of transmitting signals both dependent on and independent of its kinase activity.

Toll-like receptors (TLRs) are critical for receiving signals from molecular patterns associated with microbial pathogens to initiate innate immune responses (1–3). Interleukin 1 receptor (IL-1R) on the other hand is important for amplifying inflammatory responses triggered by microbial pathogens (4). Interestingly, signal transduction pathways mediated by the IL-1R and TLRs are very similar (5, 6). One common signaling pathway is initiated by the recruitment of the adaptor protein MyD88 to individual receptors upon ligand binding (7). MyD88 in turn is capable of recruiting IL-1 receptor-associated kinase (IRAK, also called IRAK-1) to the receptor complex (8). Upon activation and modification, IRAK-1 dissociates from the receptor complex and associates with tumor necrosis factor receptor-associated factor 6 (TRAF6) to trigger downstream signaling pathways (9–12), including the activation of NF-κB and various stress kinases such as c-Jun NH₂-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK). The apparently simple cascade of MyD88 → IRAK → TRAF6 became slightly more complex recently with a number of interesting discoveries. Starting from the top of the signaling cascade, three MyD88-like adaptor proteins, Mal (TIRAP), TRIF (TICAM-1), and TRAM (TICAM-2), were reported to play a role in mediating TLR signals (13). Although MyD88 is important in mediating signals for most TLRs and IL-1R (7), Mal helps transmit MyD88-dependent signals induced by TLR2 and TLR4 (14, 15). TRIF is critical in mediating TLR3 and TLR4 signals, particularly interferon-responsive pathways (through IRF-3) that are MyD88-independent (16, 17), and TRAM associates with TLR4 specifically to signal along a TRIF-dependent pathway (18–20).

Studies of IL-1R/TLR signals at the level of IRAKs have also made important progress over the past two years. There are a total of four IRAK family members: IRAK-1, IRAK-2, IRAK-M, and IRAK-4 (10, 21–23). As deletion of the prototypical member IRAK-1 in mice reveals a partial defect in IL-1R/TLR signaling (24, 25), it was initially speculated that other IRAK proteins might compensate for the absence of IRAK-1. However, mice lacking IRAK-M exhibit enhanced inflammatory responses induced by IL-1 or TLR ligands, suggesting that IRAK-M may instead be a negative signal regulator (26). Furthermore, we have previously reported that, in IRAK-4-deficient mice, the signals mediated by IL-1R and most TLRs are severely impaired, indicating that IRAK-4 plays an essential non-redundant role in these signaling pathways (27). IRAK-4 was shown to be primarily involved in MyD88-dependent pathways including signals triggered by IL-18 and LPS (28, 29), but the relationship between IRAK-4 and IRAK-1 and the requirement of the kinase activity of IRAK-4 in various signaling events remain to be addressed (30).

In this report, we demonstrate that IRAK-4 is an integral component of the IL-1R signaling complex induced by IL-1 stimulation. The presence of IRAK-4 is required for the recruitment of IRAK-1 to the receptor complex and for the activation and subsequent degradation of IRAK-1 protein. Reconstitution of IRAK-4-deficient cells with wild type IRAK-4, but not kinase-inactive IRAK-4, is capable of restoring IL-1 responses. Interestingly, kinase-inactive IRAK-4 still supports partial cytokine production in IL-1-stimulated mutant cells. These results provide genetic evidence that IRAK-4 is required for IRAK-1 function and that IRAK-4 can transmit signals both through phosphorylation of downstream substrates and through protein-protein interactions that are independent of its kinase activity.

MATERIALS AND METHODS

Biological Reagents—Recombinant mouse IL-1β was purchased from R&D. Polyclonal rabbit antiserum to murine IRAK-1 was raised against a peptide corresponding to the COOH-terminal amino acids 439–459 of mouse IRAK-4 protein. Polyclonal rabbit antiserum to murine IRAK-1 was raised against a peptide corresponding to the COOH-terminal amino acids 692–712 of mouse IRAK-1 protein. Anti-mouse CD121a

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Recombinant mouse IL-1β (R&D Systems, Minneapolis, Minnesota) was purchased from R&D. Polyclonal rabbit antiserum to murine IRAK-1 was raised against a peptide corresponding to the COOH-terminal amino acids 439–459 of mouse IRAK-4 protein. Polyclonal rabbit antiserum to murine IRAK-1 was raised against a peptide corresponding to the COOH-terminal amino acids 692–712 of mouse IRAK-1 protein. Anti-mouse CD121a

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IRAK-4 protein levels from total protein lysates.

**Fig. 1. IRAK-4 is required for IL-1 signals and is recruited to the IL-1R complex.** A, IL-6 cytokine production by IRAK-4-deficient cells. IL-6 cytokine production was measured in supernatants from wild type and IRAK-4-deficient EF cells with or without 24-h IL-1 treatment. Cytokine profiles are shown for a representative wild type (WT) EF cell line and three independent IRAK-4-null EF cell lines (ko1, ko2, and ko3). B, IRAK-4 recruitment to the IL-1R complex. Wild type EF cells were left untreated or stimulated with IL-1β (10 ng/ml) for the indicated periods of time. Total protein lysates were immunoprecipitated with an antibody recognizing murine IL-1RI and then subjected to gel fractionation and Western blot analysis using an IRAK-4-specific antibody. The bottom panel shows the direct Western blot analysis of IRAK-4 protein levels from total protein lysates. MEFs, mouse embryonic fibroblasts.

**Fig. 2. Effects of IRAK-4 deficiency on IRAK-1 recruitment and activation.** A, wild type and IRAK-4 knock-out EF cells were left untreated or stimulated with IL-1β (10 ng/ml) for the indicated periods of time. Total protein lysates were immunoprecipitated with an antibody recognizing murine IL-1RI and then subjected to gel fractionation and Western blot analysis using an IRAK-1-specific antibody. B, similar to A with the exception that the lysates were immunoprecipitated with an IRAK-1-specific antibody. MEFs, mouse embryonic fibroblasts.

(IL-1 receptor, type I/80) monoclonal antibody, anti-human JNK1 polyclonal antibody, and anti-Il-Bα polyclonal antibody were purchased from BD Biosciences, Santa Cruz Biotechnology, and Cell Signaling, respectively. 3T3-like immortalized mouse embryonic fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

**Cloning and Expression Vectors—**Human wild type and kinase-inactive IRAK-4 (K213A/K214A) vectors were obtained from H. Wessche (Tularik Inc.). Murine IRAK-4 CDNA was obtained by PCR with a universal mouse cDNA library as template. Expression vectors for IRAK-4 were constructed by inserting PCR-generated cDNA fragments in the mammalian retroviral expression vector, pBabe-puro. Murine IRAK-4 (K213A/K214A) was constructed using the QuikChange site-directed mutagenesis kit (Stratagene).

**Cytokine Enzyme-linked Immunosorbent Assay—**For detection of secreted IL-6 protein, MEFs (5 × 10⁵/well) were incubated overnight in 24-well plates in 10% fetal bovine serum-Dulbecco’s modified Eagle’s medium. Cells were treated for 24 h with IL-1β (10 ng/ml) in fresh medium. Supernatants were then collected and analyzed for the presence of IL-6 using a commercial enzyme-linked immunosorbent assay kit (from BD Biosciences) using recombinant mouse IL-6 as a standard.

**Transfection-based Reporter Gene Assays—**Cells were seeded at a density of 8 × 10⁴/well in 6-well plates 24 h prior to transfection. For experiments with the NF-κB-dependent endothelial leukocyte adhesion molecule promoter, cells were transfected using the polycationic transfection reagent Superfect (Qiagen) with 0.2 μg of cytomegalovirus-lacZ/0.8 μg of pELAM-luciferase reporter and the indicated amounts of expression constructs. 42 h after transfection, cells were left untreated or stimulated with 10 ng/ml IL-1β for 6 h. Cells then were washed twice with PBS and lysed in 200 μl of reporter lysis buffer (Promega) at room temperature for 30 min. Luciferase activity in 20 μl of extract was measured immediately using the luciferase assay system (Promega) and a luminometer (SpectraFluor Plus, TECAN) according to the manufacturers’ instructions. β-galactosidase activity was measured in 20 μl of extract with 4 mg/ml ONPG (o-nitrophenyl-β-D-galactopyranoside, Sigma) in a 0.067 M of sodium phosphate buffer (pH 7.5). The optical density then was measured at 405 nm. Fold activation of reporter activity was calculated for each sample by dividing the luciferase activity in the experimental sample (normalized to β-galactosidase activity) by the luciferase activity in the unstimulated control.

**Stable Transfections—**pBabe-puro (24), a murine leukemia virus-based retroviral vector, was used to transduce the target genes. 16 h prior to transfection, the packaging cell line (Phoenix) was seeded at 5 × 10⁶ cells/10-cm plate. Cells were grown to 80–70% confluency and transfected with empty pBabe-puro, pBabe-mIRAK-4, or pBabe-mIRAK-4 K213A/K214A vectors using a calcium-phosphate method. Viral supernatants were collected 48 h after transfection and then added to wild type or irak-4 knockout cells. The infected cells were grown for 24 h and then subjected to selection in the same medium containing puromycin (2 μg/ml).

**Immunoprecipitation and Western Blot Analyses—**Cells (2 × 10⁶) were seeded on 10-cm plates and incubated with or without 10 ng/ml IL-1β for the indicated amount of time. For harvesting, the plates were immediately chilled in an ice tray, washed once with ice-cold PBS, and then scraped in ice-cold PBS. The cells were centrifuged, and the cell pellet was lysed for 30 min on ice in 500 μl of lysis buffer containing 50 mM Tris, pH 8.0, 20 mM EDTA, 150 mM NaCl, 1% Triton X-100, and a protease inhibitor mixture containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, and 10 μg/ml leupeptin. The cells were spun for 15 min at 6000 rpm in an Eppendorf 5415 C centrifuge, and the solubilized lysate was collected. Lysates were preclarified with 20 μl of a 50% slurry of protein A-Sepharose or Gamma-bind Plus-Sepharose (Amersham Biosciences) for 30 min at 4°C on a rotator. Proteins were immunoprecipitated from preclarified lysates by the addition of 1–2 μl of antibody and 30 μl of Sepharose beads followed by incubation for 2–3 h at 4°C on a rotator. The samples were washed five times in lysis buffer, and the beads were resuspended in 2× SDS sample buffer. Proteins
The Role of IRAK-4 Kinase Activity in IL-1R Signaling

Results

IRAK-4 Is Recruited to the IL-1R Signaling Complex—To study the role of IRAK-4 in IL-1 signaling, three independent sets of IRAK-4-deficient embryonic fibroblasts (EF) and their wild type littermate controls were generated. As shown in Fig. 1A, the defect in IL-1-mediated IL-6 production was reproduced in all of the knock-out lines. These independent IRAK-4 knock-out lines were all utilized in the experiments in this study to minimize the contribution of clonal variation.

We have previously demonstrated that signals downstream of MyD88 (and Mal) depend on IRAK-4, whereas overexpression of TRAF6 is capable of activating NF-κB independent of IRAK-4. These results suggest that IRAK-4 functions in the signaling cascade between MyD88 and TRAF6 in a similar position as IRAK-1 (5, 8). We first investigated whether IRAK-4 is recruited to the receptor complex as MyD88 and IRAK-1. As shown in Fig. 1B, as early as 1 min after IL-1 stimulation, IRAK-4 is recruited to the IL-1R-associated complex. Interestingly, unlike IRAK-1 that quickly dissociates from the receptor complex (Fig. 2A) and is degraded (6), IRAK-4 interacts with IL-1R for up to 240 min after stimulation and the total protein level of IRAK-4 does not appear to change during this time period (Fig. 1B). In addition, a mild smearing pattern of IL-1R-associated IRAK-4 was observed at late time points after IL-1 stimulation (Fig. 1B), although the nature of this modification remains unclear.

IRAK-4 Deficiency Impairs IRAK-1 Recruitment and Activation—As IRAK-1 has been shown to interact with IRAK-4 shortly and transiently after IL-1 stimulation (23), we examined whether the absence of IRAK-4 affects the function of IRAK-1 in IL-1 signaling. Indeed, the recruitment of IRAK-1 to the IL-1R complex in response to IL-1 is severely impaired in cells lacking IRAK-4 (Fig. 2A). Subsequent to its recruitment to the IL-1R complex in wild type cells, IRAK-1 becomes phosphorylated (not easily visible in EF cells) and then degraded (readily visible in EF cells) (30). However, in the absence of IRAK-4, IRAK-1 does not undergo activation or degradation following IL-1 treatment (Fig. 2B). These results suggest that IRAK-4 is essential for the recruitment and activation of IRAK-1. It is interesting to note that the level of IRAK-1 expression is enhanced in IRAK-4-deficient cells compared with their wild type counterparts (Fig. 2B), although this enhancement does not confer compensation in IL-1-mediated responses.

The Role of IRAK-4 Kinase Activity on IL-1 Signaling Output—If IRAK-4 can function as a kinase and activate downstream substrates such as IRAK-1 (23, 31), it is possible that the kinase activity of IRAK-4 plays a role in IL-1 signal transduction. Indeed, transient overexpression of wild type IRAK-4, but not kinase-inactive IRAK-4, in three independent IRAK-4-deficient cell lines was sufficient to rescue the IL-1-induced response as measured by NF-κB-dependent reporter assays (one representative experiment is shown in Fig. 3). The intensity of the IL-1-mediated NF-κB signal was proportional to the extent of wild type IRAK-4 overexpression. In contrast, equiv-
alent levels of kinase-inactive IRAK-4 expression resulted in severely reduced IL-1 responses compared with wild type reconstitution (Fig. 3).

To further investigate the requirement of IRAK-4 kinase activity for IL-1R signaling, we generated stable lines of IRAK-4 null cells expressing vector alone, wild type IRAK-4, or kinase-inactive IRAK-4. Wild type littermate control cells expressing vector alone were utilized as the standard for IL-1 responses. First, we measured IL-1-induced cytokine (IL-6) production and found that mutant cells reconstituted with wild type IRAK-4 were capable of producing IL-6 to almost the same level as the wild type standard. On the other hand, mutant cells reconstituted with kinase-inactive IRAK-4 produced approximately one-tenth to one-fifth of the cytokine levels that cells reconstituted with wild type IRAK-4 produced (Fig. 4 A).

The level of IL-6 production induced by the kinase-inactive IRAK-4, although severely reduced, was significant considering IRAK-4 mutant cells reconstituted with vector alone pro-
duced undetectable levels of IL-6.

We confirmed the phenotype of a partial cytokine response mediated by kinase-inactive IRAK-4 by performing stable transfection experiments on all three IRAK-4 knock-out cell lines (data not shown). We also compared wild type and kinase-inactive IRAK-4 reconstitutions using pooled stably transfected cells as well as individually selected stable clones, and similar results were obtained (data not shown). Importantly, protein expression levels of wild type and kinase-inactive IRAK-4 in stable lines were characterized, and those with similar expression levels were utilized for these experiments (Fig. 4A). Finally, we also utilized human wild type and kinase-inactive IRAK-4 to reconstitute mouse IRAK-4-knock-out cells and again kinase-inactive IRAK-4 only partially reconstituted the cytokine response induced by IL-1 (Fig. 4B).

NF-κB and JNK activation have been reported to play a role in IL-6 production (32, 33). Therefore, we examined these signaling events in IRAK-4 null cells reconstituted with wild type or kinase-inactive IRAK-4. As expected, compared with wild type IRAK-4 reconstitution, the reconstitution of mutant cells with kinase-inactive IRAK-4 resulted in severely defective NF-κB and JNK activation induced by IL-1 (Fig. 4, C and D). These results suggest that the kinase activity of IRAK-4 plays an important role in IL-1 signals leading to NF-κB and JNK activation.

We also examined whether the kinase activity of IRAK-4 plays a role in the activation of IRAK-1 in response to IL-1. Compared with wild type IRAK-4 reconstitution of mutant cells, reconstitution with kinase-inactive IRAK-4 was incapable of fully restoring IRAK-1 degradation (which is dependent on its activation) after IL-1 stimulation (Fig. 4E).

**DISCUSSION**

The common signaling cascade of MyD88 → IRAK → TRAF6 is responsible for mediating signals induced by the IL-1R and most TLR family members (with the exception of TLR3 and TLR4) (34). Therefore, studies of how this signaling pathway is regulated will have therapeutic implications for many infectious diseases and inflammatory disorders. In this report, we examine the function of IRAK-4 and its interaction with IRAK-1, both being kinases that play important roles in IL-1R and TLR signaling cascades. We demonstrate that IRAK-4 is essential for IL-1-mediated signals at least partly by participating in the recruitment of IRAK-1 to the receptor complex and in the activation of IRAK-1. The kinase activity of IRAK-4 is indeed required for mediating IL-1-induced NF-κB and JNK activation and for the optimal induction of inflammatory cytokines.

In knock-out cells reconstituted with kinase-inactive IRAK-4, the only IRAK-4 molecules available for signaling complex formation lack enzymatic activity but can presumably still signal via their physical association with other signaling molecules. From our results, these kinase-independent interactions are capable of generating a partial cytokine response to IL-1 stimulation. The signals responsible for this partial response are still unknown. One possibility may lie in the kinase activity of IRAK-1 (6, 31). A small fraction of IRAK-1 molecules may be active at the receptor complex, or they may function in the cytosol in response to processed downstream signals. Alternatively, it is interesting to speculate that signaling molecules other than IRAK-1 may mediate signals independent of IRAK-4 kinase activity. These molecules may depend more on protein domain interactions and aggregation to signal. Further studies of novel IRAK-4-interacting proteins could provide important clues to this question.

Upon IL-1 stimulation, IRAK-4 interacts with the IL-1R for a sustained period of time, whereas, in contrast, IRAK-1 associates with the receptor only transiently in EF cells. Whether IRAK-4 can transmit signals from the receptor complex when IRAK-1 is absent remains to be addressed. It is possible that there are IRAK-4 substrates other than IRAK-1 that can promote IL-1 signals. Alternatively, the prolonged inclusion of IRAK-4 in the IL-1R complex may serve as a negative signal at later stages to quench ongoing inflammatory responses. Consistent with the latter possibility, the overexpression of IRAK-4 has been shown to inhibit IL-1 signals in certain cell contexts (35).

The relationship between IRAK-4 and two other IRAK family members, IRAK-2 and IRAK-M, remains unclear. It is possible that IRAK-2 or IRAK-M can interact with IRAK-4 and may act as substrates for IRAK-4. How these two kinase-inactive IRAK family members might contribute to signaling cascades in a positive manner (thereby mimicking IRAK-1 function) or as negative regulators (as demonstrated in the IRAK-M knock-out) are interesting questions to investigate. Future studies comparing mutant mice and cells lacking individual or combinations of IRAK molecules will help to clarify the molecular mechanisms involving different IRAK molecules.

Our results demonstrating the requirement for the kinase activity of IRAK-4 in IL-1R signaling are significantly different from those observed by Qin et al. (36). They concluded from their data using human kinase-inactive IRAK-4 to reconstitute a transformed adult fibroblast cell line derived from an IRAK-4-deficient patient (37) that the kinase activity of IRAK-4 is dispensable for its signaling function. The reasons for this discrepancy are unclear, but there are three major differences in our experimental systems: 1) mouse versus human; 2) embryonic fibroblasts versus adult fibroblasts; and 3) the status of cell transformation. It is possible that all three factors influenced our results. If the requirement for the kinase activity of IRAK-4 does vary between different tissues, it will be very interesting to understand the standpoint of the mechanisms of signaling as well as drug development. Further studies will help elucidate these possibilities.

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Note: The reference section includes multiple papers from the same authors, indicating a comprehensive study on the role of IRAK-4 in the immune response. The references are crucial for understanding the context and implications of the research.
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