IRAK4 kinase activity is redundant for IRAK phosphorylation and IL-1 responsiveness

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Running title: The Kinase activity of Human IRAK4

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Abstract

IL-1 stimulation leads to the recruitment of interleukin-1 receptor associated kinase (IRAK) to the IL-1 receptor, where IRAK is phosphorylated, ubiquitinated and eventually degraded. Kinase-inactive mutant IRAK is still phosphorylated in response to IL-1 stimulation when it is transfected into IRAK-deficient cells, suggesting that there must be an IRAK kinase in the pathway. The fact that IRAK4, another IRAK family member necessary for the IL-1 pathway, is able to phosphorylate IRAK in vitro suggests that IRAK4 might be the IRAK kinase. However, we now found that IRAK4 kinase-inactive mutant had the same ability as the wild-type IRAK4 in restoring IL-1-mediated signaling in human IRAK4-deficient cells, including NFκB-dependent reporter gene expression, activation of NFκB and JNK, and endogenous IL-8 gene expression. These results strongly indicate that the kinase activity of human IRAK4 is not necessary for IL-1 signaling. Furthermore, we showed that the kinase activity of IRAK4 was not necessary for IL-1-induced IRAK phosphorylation, suggesting that IRAK phosphorylation can probably be achieved either by autophosphorylation or by trans-phosphorylation through IRAK4. In supporting of this, only the impairment of the kinase activity of both IRAK and IRAK4 efficiently abolished the IL-1 pathway, demonstrating that the kinase activity of IRAK and IRAK4 is redundant for IL-1-mediated signaling. Moreover, consistent with the fact that IRAK4 is a necessary component of the IL-1 pathway, we found that IRAK4 was required for the efficient recruitment of IRAK to the IL-1 receptor complex.
Introduction

The Toll-IL-1 receptor superfamily, a large family of proteins defined by the presence of an intracellular Toll-IL-1 receptor (TIR) domain, plays crucial roles in the immune responses. This superfamily can be divided into two main subgroups based on their extracellular domains, the Immunoglobulin domain (Ig) containing receptors (1) and the Leucine Rich Repeat motif (LRR) containing receptors (2). The Ig domain subgroup includes IL-1R1, IL-18R, and T1/ST2. IL-1 has been demonstrated to be one of the key orchestrators of the immune response, eliciting a wide range of biological responses, including fever, lymphocyte activation, and leukocyte infusion to the site of injury and infection (3). The LRR subgroup consists of at least ten Toll-like receptors (TLRs) (4-7), which detects invasion of pathogens by recognizing the pathogen-associated molecular patterns, leading to the activation of innate and adaptive immune responses.

The IL-1R/TLR superfamily delivers biological activities mainly by activating the transcription of various genes in different target cells. Tremendous effort has been devoted to understanding the signaling pathways mediated by this receptor superfamily. Due to the similarities in their cytoplasmic domains, these receptors employ related yet distinct signaling components and downstream pathways. Because IL-1R was the first discovered receptor in this superfamily, the IL-1-mediated signaling pathway serves as a “prototype” for other
family members. Genetic and biochemical studies revealed that IL-1R mediates a very complex pathway, involving a cascade of kinases organized by multiple adapter molecules into sequential signaling complexes, leading to activation of the transcription factors NFκB, ATF and AP-1 (8-10). Based on studies by our group and others (11-13), we postulated a model of the IL-1 pathway (Fig. 1). Upon IL-1 stimulation, adapter molecules (including MyD88 (14) and Tollip (15) are first recruited to the IL-1 receptor, followed by the recruitment of two serine-threonine kinases, IRAK4 (16;17) and IRAK (18;19), and the adapter TRAF6 (20), resulting in the formation of the receptor complex (Complex 1). During the formation of Complex I, IRAK is phosphorylated, which creates an interface for its interaction with adapter Pellino 1 (11). The formation of Pellino 1-IRAK4-IRAK-TRAF6 causes conformational changes in the receptor complex (Complex I), releasing these signaling molecules from the receptor. The released components then interact with the membrane bound pre-associated TAK1-TAB1-TAB2 (12), resulting in the formation of Complex II (IRAK-TRAF6-TAK1-TAB1-TAB2). TAK1 and TAB2 are phosphorylated in the membrane-bound Complex II, triggering the dissociation and translocation of TRAF6-TAK1-TAB1-TAB2 (Complex III) from the membrane to the cytosol. The translocated Complex III interacts with additional factors in the cytosol, leading to TAK1 activation. The activation of TAK1 eventually leads to the activation of IκB kinase (IKK), in turn leading to the phosphorylation and degradation of IκB proteins, and liberation of NFκB to activate transcription in the nucleus (21-24). Activated TAK1 has also been implicated in the IL-1-induced activation of MKK6 and JNK (25), leading to the activation of other transcription factors, including ATF and AP1, thereby also activating gene transcription.
While the above model of the IL-1 pathway is well supported by our published studies and work from other groups, the detailed signaling mechanism for the pathway is still unclear. We recently further investigated the formation and activation of the IL-1 receptor complex (Complex I), especially regarding the mechanism of IRAK and IRAK4 in the receptor complex. IRAK is hyperphosphorylated at the receptor complex. The phosphorylation of IRAK is likely to play an important role in IL-1-mediated signaling, although the kinase activity of IRAK is dispensable for its function (19). We have previously shown that kinase-inactive mutant IRAK is still phosphorylated in response to IL-1 stimulation when it is transfected into IRAK-null cells, suggesting that there must be an IRAK kinase in the pathway (19). Recent studies indicate that another member of the IRAK family, IRAK4 is likely to function as the IRAK kinase. Severe impairment of IL-1R/TLR-mediated signaling is observed in mice lacking IRAK4 and in human patients deficient in IRAK4 (16;17;26;27). Furthermore, IRAK4 is able to phosphorylate IRAK in vitro (16). Therefore, it has been postulated that IRAK4 is recruited to the IL-1 receptor upon IL-1 stimulation, where it is activated, leading to the phosphorylation of IRAK. However, we now found that the kinase activity of human IRAK4 is dispensable for IL-1-mediated signaling. The presence of kinase activity from either IRAK or IRAK4 is sufficient to phosphorylate IRAK. Although IRAK4 is not necessary for IRAK phosphorylation, IRAK4 facilitates the recruitment of IRAK to the IL-1 receptor complex.

**Material and Methods**

**Biological reagents and cell culture.** Recombinant human IL-1β was provided by the National Cancer Institute. Anti-JNK, anti-phospho-JNK, anti-IL-1R and anti-IRAK
polyclonal antibodies were from Santa Cruz (Santa Cruz Biotechnology). Anti-IRAK4 polyclonal antibody was kindly provided by Dr. Holger Wesche (Tularik, South San Francisco, CA). 293-TK/Zeo cells(19), I1A cells(19), and human IRAK-4 deficient fibroblasts(26) were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, penicillin G (100 µg/ml), and streptomycin (100 µg/ml).

**Recombinant plasmids and transfection.** pE-selectin-luc, an NFκB-dependent E-selectin-luciferase reporter plasmid, and AP-1-dependent-luciferase reporter construct were described by Schindler and Baichwal (23). N-terminal Flag-tagged wild type, kinase-inactive (KK213AA, two lysine residues in the ATP binding pocket were mutated to alanine, abbreviated as IRAK4KD.) and truncated (1-191aa) IRAK-4 cDNA were cloned into the retrovirus vector, pBabe-puro. Transfection of the IRAK-4 deficient fibroblasts was performed using the FuGEN 6 Transfection Reagent, as recommended by the manufacturer (Roche Diagnostics, Indianapolis, IN). Transfection solution was prepared by mixing 1 µg of plasmid DNA and 3 µl of FuGEN 6 Transfection Reagent in 100 µl of serum-free medium. After incubation at room temperature for 15 min, the mixture was added to tissue culture wells containing 1 x 10^6 cells in 2 ml of complete culture medium.

**Coimmunoprecipitation and immunoblotting.** Cells that were not treated or treated with IL-1 (100 U/ml) were lysed in a Triton-containing lysis buffer (0.5% Triton X-100, 20 mM HEPES [pH 7.4], 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl₂, 10 mM NaF, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 2 mM EGTA, 20 µM aprotinin, 1 mM phenylmethylsulfonyl fluoride). Cell extracts were incubated with 1 µg of antibody or preimmune serum (negative control) for 2 h, followed by a 2-h incubation with 20 µl of protein A-Sepharose beads (prewashed and resuspended in phosphate-buffered saline at a 1:1
ratio). After incubation, the beads were washed four times with lysis buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P membranes (Millipore), and analyzed by immunoblotting.

**Reporter Assays** 2 × 10⁵ cells were transfected by the same procedure as described above with 1 µg of pE-selectin-luc, 1 µg of pSV₂-βgal, and 100 ng of each expression construct. After 48 h, the cells were split onto two 35-mm plates and stimulated with IL-1 the next day for 4 h before harvest. Luciferase and β-galactosidase activities were determined by using the luciferase assay system and chemiluminescent reagents from Promega (Madison, WI).

**Gel Shift Assays and Northern blotting** An NF-κB binding site (5’-GAGCAGAGGAAATCCGTAACTT-3’) from the IP-10 gene was used as a probe. Complementary oligonucleotides, end-labeled with polynucleotide kinase (Roche Molecular Biochemicals) and β-32P-labeled ATP, were annealed by slow cooling. Approximately 20,000 cpm of probe were used per assay. Whole cell extracts were used for the assay. The binding reaction was carried out at 4 °C for 20 min in a total volume of 20 µl containing 20 mM Hepes buffer, pH 7.0, 10 mM KCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 0.25 mM phenylmethanesulfonyl fluoride, and 10% glycerol.

For Northern analysis, total RNA was isolated by using the TRIzol reagent (GIBCO BRL). Appropriate gene-specific probes were made with a random priming kit (Amersham). Transfers to the positively charged nylon membrane Hybond-N were performed according to the procedure provided by Amersham.

**Results**
The kinase activity of human IRAK4 is dispensable for IL-1-mediated signaling

Severe impairment of IL-1R/TLR-mediated signaling is observed in mice lacking IRAK4 and in IRAK4-deficient cells derived from human patients, indicating that IRAK4 is required for IL-1-mediated signaling (16;17;26). However, it is unclear whether the kinase activity of IRAK4 is necessary for the pathway. To address this question, increasing amounts of DNA of the human wild-type (IRAK4wt) and kinase-inactive mutant IRAK4 (KK213AA, abbreviated as IRAK4KD) were co-transfected into human IRAK4-deficient cells with NFκB-dependent-E-selectine-luc and AP-1-dependent-Luc reporter constructs. As shown in Figure 2, wild-type (IRAK4wt) and the kinase-inactive mutant IRAK4 (IRAK4KD) displayed similar ability in restoring the IL-1-induced NFκB- and AP-1-dependent luciferase activity, suggesting that the kinase activity of human IRAK4 is not necessary for IL-1 signaling.

We then compared the ability of IRAK4KD (kinase-inactive mutant IRAK4) with IRAK4wt (wild-type IRAK4) in restoring IL-1-induced NFκB and JNK activation. Equal amounts of human IRAK4wt and IRAK4KD were stably co-transfected with pBaBePURO into human IRAK4-deficient cells. The puromycin-resistant clones were pooled and then either untreated or treated with IL-1 for the indicated times, followed by NFκB gel shift assay and Western analysis with anti-phospho-JNK. As shown in Figure 3, IRAK4wt (wild-type IRAK4) and IRAK4KD (kinase-inactive mutant IRAK4) restored similar levels of NFκB and JNK activation in human IRAK4-deficient cells in response to IL-1 stimulation, confirming that the kinase activity of human IRAK4 is dispensable for IL-1-mediated signaling.
We also examined IL-1-induced gene expression in human IRAK4-deficient cells stably transfected with either IRAK4wt (human wild-type IRAK4) or IRAK4KD (human kinase-inactive mutant IRAK4). As shown in Figure 4, the kinase inactive mutant IRAK4 (IRAK4KD) was able to restore similar levels of IL-8 gene expression in response to IL-1 stimulation as compared to wild-type IRAK4 (IRAK4wt). Taken together, the above results clearly demonstrate that the kinase activity of human IRAK4 is not necessary for IL-1-mediated signaling pathway.

The kinase activity of human IRAK and IRAK4 is redundant for IL-1-mediated signaling

We have previously taken a genetic approach to study IL-1-dependent signaling pathways; through random mutagenesis, we generated IL-1-unresponsive cell lines lacking specific components of the pathways from human embryonic kidney 293 cells(19). Mutant cell line I1A, which lacks both IRAK protein and mRNA, has been used effectively to study structure-function relationships of IRAK in IL-1-dependent signaling. Neither NFκB nor JNK is activated in IL-1 treated I1A cells, but these responses are restored in I1A-IRAK cells, indicating that IRAK is required for both. However, the kinase activity of IRAK is not required for IL-1-dependent signaling, since kinase-dead IRAK mutants were still able to restore IL-1 responsiveness. The fact that the kinase-inactive mutant IRAK is still phosphorylated in response to IL-1 stimulation when it is transfected into I1A cells, suggesting that there must be an IRAK kinase in the pathway (19). As IRAK4 was able to phosphorylate IRAK in vitro, it was suggested that IRAK4 might be the IRAK kinase. To
examine the role of IRAK4 in IL-1-induced IRAK phosphorylation, we examined IL-1-induced IRAK modification in human IRAK4-deficient cells stably transfected with wild-type IRAK4 and kinase-inactive IRAK4 mutant. As shown in Figure 5, IRAK was not phosphorylated or ubiquitinated upon IL-1 stimulation in IRAK4-deficient cells transfected with vector DNA (Vector). Transfection of wild-type IRAK4 (IRAK4wt) into the IRAK4-deficient cells restored IL-1-induced IRAK phosphorylation and ubiquitination in these cells. Interestingly, IL-1-induced IRAK modification was also restored in IRAK4-deficient cells transfected with the kinase-inactive mutant IRAK4 (IRAK4KD), indicating that the kinase activity of human IRAK4 is not necessary for IL-1-induced IRAK phosphorylation. The observed IRAK modification in IRAK4-deficient cells stably transfected with the kinase-inactive mutant IRAK4 could very well be due to IRAK auto-phosphorylation in response to IL-1 stimulation.

Taken together, the above results showed that although both human IRAK and IRAK4 are required for IL-1 signaling, their kinase activity are not necessary for the pathway, suggesting that kinase activity of human IRAK4 and IRAK might be redundant for IL-1 signaling. To test this hypothesis, we co-transfected increasing amounts of DNA of IRAK4KD (kinase-inactive mutant IRAK4) into I1A cells either with wild-type IRAK (IRAKwt) or kinase-inactive mutant IRAK (K239A, abbreviated as IRAKmt). Interestingly, whereas the kinase-inactive mutant IRAK4 (IRAK4KD) had minimum effect on E-selectin-promoter-driven luciferase activity when it is co-transfected with wild-type IRAK (IRAKwt) into the I1A cells, the IRAK4 mutant (IRAK4KD) efficiently inhibited the luciferase activity upon its co-transfection with the kinase-inactive mutant IRAK (IRAKmt) into the I1A cells (Fig. 6). These results showed that only the impairment of the kinase activity of both IRAK and
IRAK4 efficiently abolished the IL-1 pathway, suggesting that the kinase activity of human IRAK and IRAK4 is probably indeed redundant for IL-1-mediated signaling.

**IRAK4 facilitates the recruitment of IRAK to the IL-1 receptor complex**

Previous studies show that upon IL-1 stimulation, the adapter molecules MyD88 and Tollip are recruited to the IL-1 receptor, followed by recruitment of two serine-threonine kinases, IRAK4 and IRAK, and the adapter TRAF6, resulting in formation of the receptor complex (Complex 1) (11;12;28). As shown in Figure 7A, IRAK4 was still efficiently recruited to the receptor in both IRAK-null I1A and MyD88-null I3A cells, indicating that the recruitment of IRAK4 does not require MyD88 or IRAK. On the other hand, IRAK was not recruited to the IL-1 receptor in MyD88-null I3A cells, confirming that MyD88 is responsible for recruiting IRAK to the receptor upon IL-1 stimulation (Fig. 7A). Taken together, these results indicate that The IL-1-induced recruitment of IRAK4 to the IL-1 receptor is upstream of MyD88.

While the above results clearly indicate that the recruitment of IRAK4 to the IL-1 receptor is upstream and independent of IRAK, we examined the role of IRAK4 in the recruitment of IRAK to the IL-1 receptor. As shown in Figure 7B, while small amount of IRAK was recruited to the IL-1 receptor in IRAK4-deficient cells upon IL-1 stimulation, transfection of IRAK4 facilitated the recruitment of IRAK to the receptor in these cells.

**Discussion**

In this manuscript, we showed that IRAK4 kinase-inactive mutant had the same ability as the wild-type IRAK4 in restoring IL-1-mediated signaling in IRAK4-deficient cells, indicating that the kinase activity of IRAK4 is not necessary for the IL-1 pathway. The fact that only
the impairment of the kinase activity of both IRAK and IRAK4 efficiently abolished the IL-1 pathway suggests that the kinase activity of IRAK and IRAK4 is probably redundant for IL-1-mediated signaling. Furthermore, we showed that while the kinase activity of IRAK4 was not necessary for IL-1-induced IRAK phosphorylation, IRAK4 facilitated the recruitment of IRAK to the IL-1 receptor complex. Based on these results, we hypothesize that upon IL-1 stimulation, through receptor adapter molecules, IRAK4 is recruited to the IL-1 receptor, which then facilitates the recruitment of IRAK to the receptor, where IRAK is hyperphosphorylated. The IL-1-induced IRAK phosphorylation can be achieved either by autophosphorylation or by trans-phosphorylation through IRAK4. The phospho-IRAK then interacts with receptor proximal signaling components TRAF6 and Pellino1 and is subsequently dissociated from the IL-1 receptor, leading to the activation of TAK1 and IKK, and activation of NFκB.

This discovery of the redundant kinase activity of IRAK and IRAK4 is not only very important for understanding the basic mechanism of the IL-1 pathway, but also has a major impact on the development of anti-inflammatory drugs. Much effort has been devoted towards developing small molecules drugs that can specifically inhibit IRAK4 kinase activity. The results presented here indicate that it is necessary to search for drugs that can inhibit the kinase activity of both IRAK and IRAK4 in order to efficiently block IL-1-induced IRAK phosphorylation and subsequent signaling events.

One interesting observation from this study is that IRAK4 is recruited to the IL-1 receptor in MyD88- and IRAK-deficient cells upon IL-1 stimulation, indicating that the recruitment of IRAK4 is not only IRAK-independent but also MyD88-independent. It is possible that
IRAK4 is directly recruited to the IL-1 receptor without involvement of adaptors. However, IRAK4 does not contain any sequence similarity to the Toll domain. It is likely that IRAK4 uses an adaptor molecule other than MyD88 to interact with the IL-1 receptor. One possible candidate is adaptor Tollip, since it is shown to interact with the IL-1 receptor and IRAK4 upon IL-1 stimulation. Another candidate is adaptor TIRAP/Mal, which is shown to cooperate with MyD88 to mediate Toll-like receptor-mediated signaling(29). The normal IL-1 signaling observed in TIRAP/Mal-deficient mice could be due to compensatory function between TIRAP/Mal and MyD88.

Our results demonstrating the redundancy of the kinase activity of human IRAK4 and IRAK in IL-1R signaling are significantly different from those observed by W. Yeh’s group (personal communication) in the mouse system. By reconstituting IRAK4-deficient mouse embryonic fibroblasts, they showed that the kinase activity of mouse IRAK4 is required for the optimal transduction of IL-1-induced signals, although they found that IRAK4 is capable of mediating some NFκB activation. The reasons for such discrepancy are unclear. One major difference in our experimental systems is mouse versus human. Another significant difference is cell type, embryonic fibroblasts versus adult fibroblasts, although consistent results are observed between human embryonic kidney 293 cells and human fibroblasts in our experiments. Therefore, it is more likely that the requirement of kinase activity of IRAK4 for the IL-1 signaling might vary from mouse to human, which will greatly affect the strategy in developing small molecules drugs for human patients.
ACKNOWLEDGMENTS

We thank Dr. Holger Wesche for polyclonal antibody against IRAK4. This work was supported by NIH grant GM 600020 to X.L.

Reference List


Figure Legends:

**Fig. 1. Model of IL-1-mediated signaling pathway.**

**Fig 2. Complementation of human IRAK4-deficient cells.** Human IRAK4 deficient fibroblasts were co-transfected transiently with E-selectin-Luc (A) or AP-1-dependent Luc (B) with increasing amounts of human wild-type, kinase-inactive or truncated IRAK4 retroviral expression constructs. Thirty-six hours later, the cells were either untreated or stimulated for 6 h with IL-1 (100 U/ml). Luciferase activities were normalized to β-galactosidase. Data are presented as fold induction of luciferase activity in the treated cells. The experiments were repeated four times. Shown are the data from a typical experiment. Levels of the transfected flag-tagged IRAK4 [IRAK4WT, IRAK4KD and IRAK4(1-191)] are shown by Western analysis of whole cell extracts with anti-flag antibody [IRAK4(Anti-flag)] and actin was used as a loading control (Actin).

**Fig 3. IL-1-induced activation of NF-κB and JNK.** (A) NF-κB gel shift assay. Cell extracts were made from untreated or IL-1 treated (100U/ml for 15 or 30 minutes) human IRAK-4-deficient fibroblasts stably transfected with empty vector, human wild-type or...
kinase-inactive IRAK4. The NF-κB binding site from the IP-10 gene was used as a probe. The two bands in the gel shift assay are due mainly to p50-p65 heterodimers (bottom) and p65-p65 homodimers (top). (B) JNK activation induced by IL-1. Whole-cell lysates were prepared from untreated or IL-1 treated (100U/ml for 15 or 20 minutes) human IRAK-4-deficient fibroblasts stably transfected with empty vector, wild-type or kinase-inactive IRAK4 and subjected to western blot analysis using anti-phospho-c-Jun and anti-JNK antibodies (loading control).

**Fig 4. Northern analysis of IL-8 gene expression.** Total RNAs were made from human IRAK4 deficient fibroblasts stably transfected with empty vector, wild-type or kinase-inactive IRAK4 untreated or treated with IL-1 (100 U/ml for 1 to 4 hours). Human IL-8 cDNA was used as a probe, and the signals were normalized after reprobing with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Levels of the transfected flag-tagged IRAK4 (IRAK4WT and IRAK4KD) are shown by Western analysis of whole cell extracts with anti-flag antibody [IRAK4(Anti-flag)] and actin was used as a loading control (Actin).

**Fig 5. Western analysis of IL-1-induced IRAK phosphorylation and ubiquitination.** Human IRAK4 deficient fibroblasts stably transfected with control vector DNA, wild-type or kinase-inactive IRAK4 were untreated or treated with IL-1 (100 U/ml for 30 or 60 minutes). Cell extracts were analyzed by the Western procedure with anti-IRAK. Levels of the transfected flag-tagged IRAK4 (IRAK4WT and IRAK4KD) are shown by Western analysis of whole cell extracts with anti-flag antibody [IRAK4(Anti-flag)] and actin was used as a loading control (Actin). U-IRAK: Ubiquitinated IRAK. P-IRAK: phosphorylated IRAK.
Fig 6. Kinase-dependent E-selectin luciferase activation by IL-1 in the IRAK-deficient I1A cells. Wild-type or mutant IRAK (100 ng) and increasing amounts of kinase-inactive IRAK4 expression constructs were co-transfected with E-selectin-luc (100 ng) into the human IRAK-deficient I1A cells. The transfected cells were untreated or treated with IL-1 (100U/ml for 6 hours), followed by luciferase reporter assay. Data are presented as fold induction of luciferase activity in the treated cells. Shown are the averages from three independent experiments. Cells transfected with vector DNA (100 ng) and E-selectin-luc DNA (100 ng) only were used as controls. Levels of the transfected flag-tagged IRAK4KD and IRAK (IRAKwt and IRAKmt) are shown by Western analysis of whole cell extracts with anti-flag antibody [IRAK4(Anti-flag)] and anti-IRAK (IRAK), and actin was used as a loading control (Actin).

Fig 7. IL-1-induced IL-1R immune complex. (A) Extracts of 293-WT, I1A, I2A or I3A, untreated or stimulated with IL-1 (100 units/ml for 10 or 20 minutes), were immunoprecipitated with anti-IRAK-4 followed by Western analyses with antibodies against IL-1R, MyD88 and IRAK. The expression of IRAK and MyD88 in wild-type 293 cells (WT) and mutant cell lines (I1A, I2A and I3A) are shown by Western analysis of whole cell extracts (WCE) with anti-IRAK (IRAK) and anti-MyD88 (MyD88). (B) Extracts of IRAK-4 deficient fibroblasts transfected with control vector DNA or wild-type IRAK4 were untreated or stimulated with IL-1 (100 units/ml for 30 or 60 minutes), and then immunoprecipitated with anti-IRAK followed by Western analyses with antibodies against IL-1R. The expression of IRAK4 in the transfected cells is shown by Western analysis of whole cell extracts with anti-IRAK4 (IRAK4).
Fig. 1
Fig. 2A

NFκB Activation

Fold Induction

IRAK4 wt

IRAK4 KD

IRAK4 (1-191)

0

50ng

100ng

250ng

500ng

0

IκB kinase

ACTIN

IRAK4 (Anti-FLAG)
Fig 2B

**AP1 Activation**

![Graph showing AP1 Activation](image)

**Legend:**
- IRAK4 WT
- IRAK4 KD
- IRAK4(anti-flag)
- Actin

- 0
- 50ng
- 100ng
- 250ng
- 500ng

Fold Induction

[Graph with bars representing fold induction for IRAK4 WT and IRAK4 KD with different concentrations of IRAK4(anti-flag)]
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Fig. 3

**NFκB**

**P-JNK**

**JNK**
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**Fig. 4**

**IL-8**

**GAPDH**

**IRAK4(Anti-FLAG)**

**ACTIN**
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Fig. 5
Fig. 6
IP: anti-IRAK4

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- IL-1R
- MyD88
- IRAK

WCE

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- IRAK
- MyD88

Fig. 7A
Fig. 7B
IRAK4 kinase activity is redundant for IRAK phosphorylation and IL-1 responsiveness

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