IRAK4 Kinase Activity Is Redundant for Interleukin-1 (IL-1) Receptor-associated Kinase Phosphorylation and IL-1 Responsiveness*

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Interleukin-1 (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. However, we now found that the 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 the 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, the activation of AP-1, 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 support 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.

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 Ig-containing receptors (1) and the leucine-rich repeat motif-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 infusions to the site of injury and infection (3). The leucine-rich repeat subgroup consists of at least 10 Toll-like receptors (TLRs) (2, 4–7), which detect 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 the activation of the transcription factors NFκB, activating transcription factor, 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 I). 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 preassociated 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, in turn leading to the phosphorylation and degradation of IκB proteins and liberation of NFκB to activate transcription in the
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 activating transcription factor and AP1, thereby also activating gene transcription.

Although 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

**Fig. 1.** Model of IL-1-mediated signaling pathway. IKK, IκB kinase.

**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 units/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 NFκB and JNK. A, NFκB gel shift assay. Cell extracts were made from untreated or IL-1-treated (100 units/ml) human IRAK-4-deficient fibroblasts stably transfected with empty vector, human wild-type, or kinase-inactive IRAK4. The NFκB 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 (100 units/ml) 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 (P-JNK) and anti-JNK (JNK) antibodies (loading control).
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.

MATERIALS 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-IRAK 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 U/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 Woronicz et al. (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 FuGENE 6 transfection reagent, as recommended by the manufacturer (Roche Diagnostics). Transfection solution was prepared by mixing 1/10 g of plasmid DNA and 3/5 l of FuGENE 6 transfection reagent in 100/10 ml of serum-free medium. After incubation at room temperature for 15 min, the mixture was added to tissue culture wells containing 1/10 106 cells in 2 ml of complete culture medium.

Coimmunoprecipitation and Immunoblotting—Cells that were not treated or treated with IL-1 (100 units/ml for 1–4 h). 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. 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 units/ml for 1–4 h). 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 units/ml for 30 or 60 min). 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.
buffer, separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and analyzed by immunoblotting.

Reporter Assays—2 x 10^5 cells were transfected by the same procedure as described above with 1 μg of pB-Selectin-luc, 1 μg of pSN-β-galactosidase, 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 NFκB binding site (5'-GAGGAGGGAAAATTCGTAATT-3') from the IP-10 gene was used as a probe. Complementary oligonucleotides, end-labeled with polynucleotide kinase (Roche Applied Science) and [γ-32P]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 μM Hepes buffer, pH 7.0, 10 mM KCl, 0.1% Nonidet P-40, 0.5 mM diithiothreitol, 0.25 mM phenylmethanesulfonfyl fluoride, and 10% glycerol.

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

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 (IRAK4KD) were co-transfected into human IRAK4-deficient cells 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 Fig. 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 Fig. 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 with 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
or 60 min) and then immunoprecipitated (IP).

The expression of IRAK and MyD88 in wild-type 293 cells was analyzed by immunoprecipitation with anti-IRAK-4 followed by Western analysis with antibodies against IL-1R, MyD88, and IRAK. The expression of IRAK in IRAK-deficient cells transfected with vector DNA or wild-type IRAK4 facilitated the recruitment of IRAK 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.

Although 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 Fig. 7B, whereas a 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 report, 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, suggesting that the kinase activity of human IRAK and IRAK4 is probably indeed redundant for IL-1-mediated signaling. Further-more, we showed that although 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 facilitates the recruitment of IRAK to the receptor, leading to the phosphorylation of IRAK, which results in the formation of the receptor complex (Complex I) and is probably indeed redundant for IL-1-mediated signaling.

**The Kinase Activity of Human IRAK4**

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 is not necessary for the pathway, suggesting that the 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, although 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.

**IP: anti-IRAK4**

**WCE**

**IP: anti-IRAK**

**DISCUSSION**

In this report, 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, suggesting that the kinase activity of human IRAK and IRAK4 is probably indeed redundant for IL-1-mediated signaling. Further-more, we showed that although 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 IκB kinase and the 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 toward developing small molecule 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 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-dependent but also MyD88-independent. It is possible that IRAK4 is directly recruited to the IL-1 receptor without the 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 and IRAK4 upon IL-1 stimulation. Another candidate is adaptor Tollip since it is shown to interact with the IL-1 receptor. One possible domain. It is likely that IRAK4 uses an adaptor molecule other than MyD88 to interact with the IL-1 receptor. However, IRAK4 is not only IRAK-independent but also MyD88-inde-

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