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IRAK-4-dependent Degradation of IRAK-1 is a Negative Feedback Signal for TLR-mediated NF-κB Activation

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The activation of interleukin 1 receptor-associated kinase (IRAK)-1 is a key event in the transmission of signals from Toll-like receptors (TLRs). The catalytic activity of the protein kinase is not essential for its ability to activate nuclear factor (NF) κB, because transfection of a kinase-dead mutant of IRAK-1 (*IRAK-1KD*) is able to activate NF-κB in HEK293T cells. In the present study, we observed that the effect of *IRAK-1KD* was impaired by simultaneous expression of IRAK-4. The effect of IRAK-4 was accompanied by the phosphorylation and degradation of *IRAK-1KD*. Expression of *IRAK-4KD* instead of IRAK-4 did not cause these events. In IRAK-4-deficient Raw264.7 macrophages that were prepared by introducing short-hairpin RNA probes, the basal level of IRAK-1 was increased markedly. Stimulation of these cells with TLR ligands did not cause the degradation of IRAK-1, which was clearly observed in the parent cells. These results suggested that the expression of IRAK-4 alone is sufficient to cause the degradation of IRAK-1; the autophosphorylation of IRAK-1 is not necessary to terminate the TLR-induced activation of NF-κB. IRAK-4 has an ability to induce the degradation of IRAK-1 in addition to its role as an activator of IRAK-1.

Key words: IRAK, LPS, NF-κB, phosphorylation, TLR.

Abbreviations: IFN, interferon; IRF, IFN regulatory factor; IKK, IκB kinase; IL, interleukin; IRAK, IL-1R-associated kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NEMO, NF-κB essential modulator; shRNA, short-hairpin RNA; TIR, Toll-IL1 receptor homology domain; TLR, Toll-like receptor; TNF, tumour necrosis factor; TRAF, TNF receptor-associated factor; TRIF, TIR domain-containing adaptor inducing IFN-β.

The Toll-like receptor (TLR) family plays a central role in the innate immune system. TLR recognizes conserved microbial products, such as lipopolysaccharide (LPS), peptidoglycan, flagellin and unmethylated CpG motifs in bacterial DNA (*1*). All TLRs have a Toll-interleukin-1 (IL-1) receptor homology domain (TIR) in the cytoplasmic tail (*2*, *3*). Upon receptor ligation, TLR recruits a TIR-containing adaptor, MyD88, *via* TIR/TIR interaction. MyD88 then associates with members of the IL-1 receptor-associated kinase (IRAK) family. Tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) also interacts with MyD88/IRAKs to activate the IκB kinase (IKK) complex, consisting of IKKα, IKKβ and NEMO/IKKγ (*4*). The IKK complex phosphorylates IκB, resulting in the ubiquitination and subsequent degradation of IκB, which binds to NF-κB in the cytoplasm and thereby inhibits the nuclear translocation of this transcription factor. This MyD88-dependent pathway is common in TLR signalling, with the possible exception of TLR3.

The mammalian family of IRAK molecules has four members: IRAK-1, IRAK-2, IRAK-M and IRAK-4 (*5*). Among these, only IRAK-1 and IRAK-4 possess protein kinase activity. IRAK-M is considered to be a negative regulator, because mice deficient in this molecule exhibit enhanced inflammatory responses to IL-1 and TLR ligands (*6*). By contrast, IRAK-1 and IRAK-4 play positive regulatory roles in the IL-1 and TLR signalling. The phenotype of IRAK-4-deficient mice indicates an essential and non-redundant role of IRAK-4 (*7*). Deficiency of IRAK-1 impairs but does not completely abolish IL-1 actions (*8*), suggesting that other members of the IRAK family may compensate for the functions of IRAK-1. The protein kinase activity of IRAK-1 is not necessarily required for its functions, because overexpression of a kinase-inactive mutant of IRAK-1 (*IRAK-1KD*) induces the activation of NF-κB (*9*, *10*). However, a recent finding revealed that direct phosphorylation of IRF7 by IRAK-1 is indispensable for interferon (IFN) α production in response to TLR7 and TLR9 ligands (*11*, *12*).

A stimulus-induced interaction between IRAK-1 and IRAK-4 is important in TLR signalling, in which MyD88 acts as a scaffold protein for the interaction (*13*). This interaction enables IRAK-4 to phosphorylate IRAK-1, which in turn causes autophosphorylation and activation of IRAK-1 itself. The phosphorylated form of IRAK-1 loses affinity for MyD88 and gains interaction with TRAF-6, a downstream molecule leading to the activation

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of NF-κB (*14*, *15*). In the present study, we examined whether the autophosphorylation of IRAK-1 is necessary for the degradation of IRAK-1. Transfection of *IRAK-1KD* to HEK293T cells activated NF-κB, as reported previously (*9*, *10*). Simultaneous transfection of IRAK-4 produced a marked decrease in NF-κB activity, which was accompanied by the phosphorylation and degradation of *IRAK-1KD*. The results suggested that the phosphorylation by IRAK-4 is sufficient to cause degradation of IRAK-1 to prevent the TLR-induced activation of NF-κB. We also prepared an IRAK-4-deficient macrophage cell line using vector-based short-hairpin RNA (shRNA) probes. In these cells, a prominent increase in the basal level of IRAK-1 was observed. Stimulation of these cells with TLR ligands did not cause the degradation of IRAK-1, which was clearly observed in the parent cells. These results indicate that IRAK-4 has the ability to regulate negatively the MyD88-dependent pathway by inducing the degradation of IRAK-1, in addition to its role as an activator of IRAK-1.

MATERIALS AND METHODS

Materials—LPS from *Escherichia coli* (serotype 0111:B4) was purchased from Sigma (St. Louis, MO, USA). Polyclonal antibodies against IRAK-1 and IRAK-4 were prepared by immunizing rabbits with a recombinant C-terminal peptide (636 to the end) of IRAK-1 and C-terminal peptide (391 to the end) of IRAK-4, respectively. Polyclonal anti-IκB-β antibody was purchased from Santa Cruz Biotechnology. (Santa Cruz, CA, USA). CpG DNA (Cy3-oligo with the sequence: TCCATGACGTTCCTGATGCT) was synthesized by Hokkaido System Science (Sapporo, Japan). Poly I:C was purchased from GE Healthcare (formerly Amersham Bioscience, Piscataway, NJ, USA). Antibodies against phospho-Erk1/2 (Thr202/Tyr204), phospho-p38 mitogen-activated protein (MAP) kinase and phospho-IKKα/β (Ser180/ser181) were obtained from Cell Signaling Biotechnology (Beverly, MA, USA). A synthetic lipopeptide based upon the full-length macrophage-activating lipopeptide-2 kDa (Malp-2) was prepared with dipalmitoyl-*S*-glyceryl cysteine, as described previously (*16*).

Cell Culture—Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium. Raw264.7 cells were cultured in RPMI1640 medium fortified with glucose to a concentration of 4.5 g/l. All media were supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin G and 100 mg/ml streptomycin, and maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Plasmids—Complementary DNA of wild-type human IRAK-1 was amplified by reverse transcriptase–polymerase chain reaction (RT-PCR) with primers based on the sequences of GenBank accession number L76191 using human monocyte total RNA as a template. The cDNA was subcloned into pFLAG-CMV5 (Sigma) for expression in mammalian cells. pCMV6-myc-IRAK-4, pEFBOS-FLAG-TLR4 and pEFBOS-FLAG-His-MD-2 were kind gifts from Dr. T. Seya (Hokkaido University, Japan). Kinase-inactive mutants of IRAK-1 (*K239S*; *IRAK-1KD*) and IRAK-4 (*KK213AA*; *IRAK-4KD*) were generated using a QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

Reporter Gene Assay for NF-κB—HEK293 and HEK293T cells in 24-well plates were transfected with 50 ng of an NF-κB reporter plasmid (Stratagene), 10 ng of sea pansy luciferase reporter plasmid (pRL-TK vector; Promega, Madison, WI, USA) as an internal control and various amount of each expression construct using FuGENE 6 (Roche, Indianapolis, IN, USA). The total amount of DNA transfected was kept constant by supplementation with empty vectors. Twenty-four hours after transfection, cells were lysed and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega). NF-κB-dependent induction of a luciferase reporter gene in the presence of IRAK-4 was calculated as relative activity (%) to that in the absence of IRAK-4.

Immunoprecipitation and Western Blot Analysis—HEK293T cells in 24-well plates were transiently transfected with appropriate plasmids using FuGENE6. Twenty-four hours after transfection, the cells were lysed in lysis buffer containing 25 mM Tris–HCl (pH 7.6), 0.5% NP-40, 150 mM NaCl, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM ethylene diamine tetra-amino acid (EDTA), 0.1% bovine serum albumin (BSA), 20 mM sodium fluoride, 1 mM phenylmethylsulphonyl fluoride, 2 μM leupeptin, 20 μM p-amidinophenylmethylsulphonyl fluoride and 1 mM *N*-ethylmaleimide. Raw264.7 cells were lysed with the same lysis buffer after incubation with various TLR ligands. The cell lysates were centrifuged at 15,000 r.p.m. for 10 min. Aliquots of the resultant supernatants were incubated with anti-IRAK-1 antibody immobilized on protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) at 4°C for 1 h with constant rotation. The Sepharose beads were spun briefly in a microfuge and washed four times with ice-cold lysis buffer without protease inhibitor and BSA. The immunoprecipitates were heated at 100°C for 3 min in 30 μl of sample buffer, consisting of 62.5 mM Tris (pH 6.8), 1% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.02% bromophenol blue. The proteins were separated by SDS–PAGE on 7.5% slab gels and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skimmed milk or 3% BSA, incubated with appropriate antibodies and visualized by enhanced chemiluminescence (Perkin–Elmer, Norwalk, CT, USA).

RNA Interference—Two sets of oligonucleotides were cloned into the pH1 vector to express IRAK-4 siRNA hairpins downstream of the human H1 RNA promoter, as described previously (*17*). The following sequences were used: 5′-AAGGGTGGAAGAAATTAGCAG-3′ and 5′-AAGGAGATTCGAAGCCTTACT-3′. Raw 264.7 cells ($5–10 \times 10^6$ cells) were transfected by electroporation in a 300 μl final volume at 250 V/950 μF (Gene Pulser II, Bio-Rad). Twenty-four hours after transfection, puromycin (7 μg/ml) was added to the cells for selection, and incubation was continued for several days. The resistant colonies were replated to 96-well plates, at 0.5–1.0 cells/well, and cultured for several additional weeks to obtain monoclonal IRAK-4-deficient cells. To determine

the efficiency of gene silencing, total RNA was isolated with RNeasy (Qiagen, Hilden, Germany), and mRNA was assessed by RT-PCR. Control cells were prepared with empty pH1 vector in the same way.

RESULTS

IRAK-4-dependent Degradation of IRAK1—Activation of NF-κB is known to accompany the degradation of IκB, which binds to NF-κB in the cytoplasm and thereby inhibits the nuclear translocation of the transcription factor. Exposure of the macrophage cell line Raw264.7 to LPS, a TLR4 ligand, induced the degradation of IκB and IRAK-1 within 15 min (Fig. 1). IκB returned almost to basal levels at 60 min, whereas the decreased level of IRAK-1 was maintained throughout this period. The slower migrating band on SDS–PAGE detected with anti-IRAK-1 antibody may be the phosphorylated form of IRAK-1 (*18*). The density of this band varied from experiment to experiment for unknown reason, and the band is sometimes too faint to be visualized by a standard period of exposure (see Fig. 2B for example). However, a longer exposure of the blots reproducibly shows the immediate appearance of this band and its development up to 60 min after stimulation.

In order to determine the role of IRAK-4 in IRAK-1 degradation, we prepared Raw264.7 cells that are defective in IRAK-4 (ΔIRAK-4 cells). Raw264.7 cells were transfected with shRNA vector targeting IRAK-4 by electroporation. The cells showing resistance to puromycin were replated to 96-well plates at 0.5–1.0 cells/well to obtain IRAK-4-deficient colonies. As shown in Fig. 2A, no detectable expression of IRAK-4 was observed in the ΔIRAK-4 cells. The cells showed a higher level of IRAK-1 in the quiescent state, and no degradation of IRAK-1 was observed, even after the addition of LPS. In these cells, phosphorylation of IKK and degradation of IκB by LPS were attenuated and delayed.

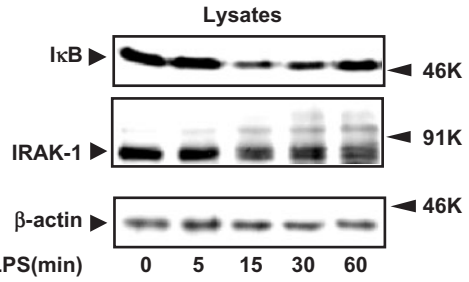

Fig. 1. **LPS-induced degradation of IRAK-1 and IκB in macrophages.** Raw264.7 cells were stimulated with LPS (100 ng/ml) for the indicated times. Whole-cell lysates were prepared and analysed by western blotting with antibodies against IκB, IRAK-1 and β-actin. The mobility of molecular markers is shown in the right of the figures.

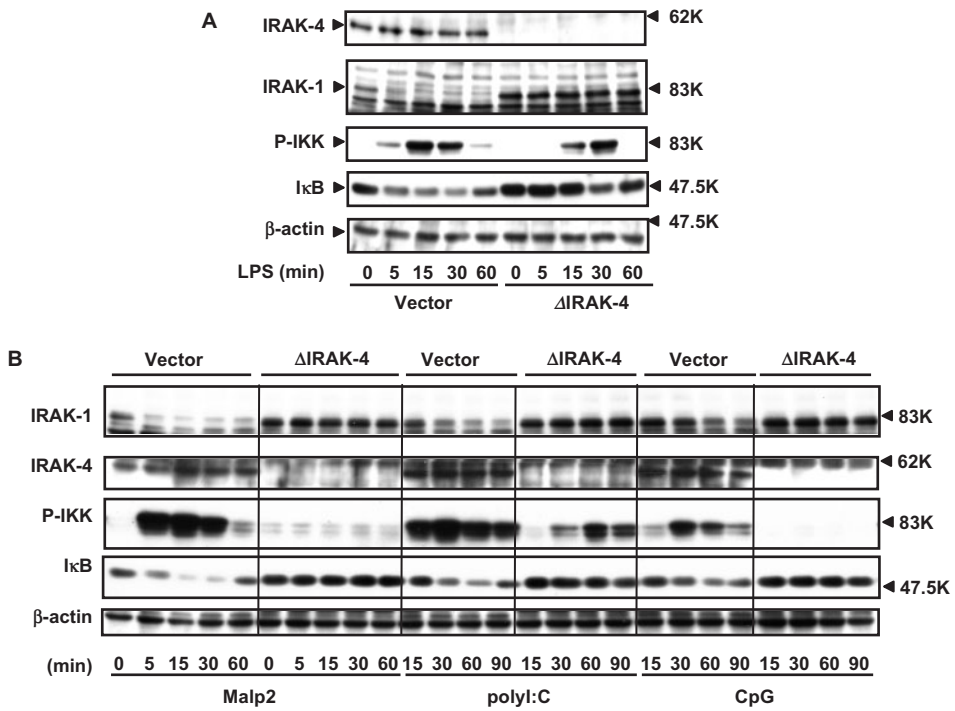

Fig. 2. **Requirement of IRAK-4 for TLR-induced IRAK-1 degradation in macrophages.** RAW264.7 cells were transfected with two sets of pH1 vector producing shRNA against IRAK-4 (10 μg each, ΔIRAK-4 cells) by electroporation. Control cells were transfected with empty vector (20 μg, Vector). After 24 h, 7 μg/ml of puromycin was added and the cells were cultured for several weeks, changing the medium every 5 days. The resistant colonies were plated in fresh medium without puromycin and cultured for an additional 24 h. In (A), the cells were stimulated with 100 ng/ml LPS. In (B), the cells were stimulated with 0.1 μM Malp2, 25 μg/ml poly(I-C) or 0.2 μM CpG. After stimulation for the indicated times, cell lysates were prepared and analysed by western blotting with antibodies against IRAK-1, IRAK-4, pIKK, IκB and β -actin. Similar results were obtained with another clone of ΔIRAK-4 cell. The mobility of molecular markers is shown in the right of the figures.

A similar delayed time course has been reported in macrophages from IRAK-4 knockout mice (*7*). It is well documented that LPS activates two signalling pathways, one of which is MyD88/TIRAP dependent and the other TRIF/TRAM dependent. The former is activated immediately after LPS challenge and the latter is activated subsequently (*2*). Therefore, the delayed activation of IKK suggested that the deficiency of IRAK-4 abolished selectively the immediate response that depends on MyD88. In agreement with this speculation, neither Malp2 (a TLR2 ligand) nor CpG (a TLR9 ligand), both of which are known to activate exclusively the MyD88-dependent pathway, elicited any responses in ΔIRAK-4 cells (Fig. 2B). The IRAK-4 deficiency did not abolish but markedly attenuated the effects of polyI:C (a TLR3 ligand). This was surprising, because TLR3 signalling is known to utilize preferentially the TRIF/TRAM (TICAM-1/TICAM-2)-dependent pathway (*19*, *20*). However, a similar impairment of TLR3-induced responses has already been reported in IRAK-$4^{-/-}$ mice and also in IRAK-4-deficient patients (*7*). Although the mechanism of this impairment is, as yet, unclear, the results in Fig. 2 indicated that the degradation of IRAK-1 is dependent on the presence of IRAK-4.

Inhibition of NF-κB by IRAK-4 Overexpression—In order to examine the role of IRAK-4-induced degradation of IRAK-1 in TLR signalling, we utilized an NF-κB reporter assay system in HEK293T cells. The introduction of wild-type IRAK-1 to these cells induced the NF-κB-dependent expression of a luciferase reporter gene (Fig. 3A). In agreement with previous reports (*9*, *10*), the kinase-inactive form of IRAK-1 (*IRAK-1KD*) induced a similar effect to that of the wild type (Fig. 3B). The effects of both IRAK-1 and *IRAK-1KD* were attenuated by coexpression of IRAK-4. The *IRAK-4KD* had no effect on NF-κB activation, indicating that the inhibitory effect of IRAK-4 is dependent on its protein kinase activity. The effect of IRAK-4 on the breakdown of *IRAK-1KD* is shown in the middle panel of Fig. 4A. Transfection of IRAK-4 effectively decreased the amount of *IRAK-1KD*. *IRAK-4KD* had no effect on the degradation. The result suggested that IRAK-4-dependent degradation of *IRAK-1KD* was responsible for its effect on NF-κB activity. When the smaller amounts of IRAK-4 were transfected, the slower migrating band of *IRAK-1KD* was observed. The band was considered to be the phosphorylated form of *IRAK-1KD*, because treatment with alkaline phosphatase abolished this band (Fig. 4B). The phosphorylated form was hardly detectable when the larger amounts of IRAK-4 were transfected, probably because of the degradation of *IRAK-1KD*. One possible mechanism for the decreased level of *IRAK-1KD* is the ubiquitination and subsequent proteasomal degradation of the protein. Unexpectedly, however, *IRAK-1KD* was highly ubiquitinated even without co-transfection of IRAK-4 (Fig. 4A, upper panel). A proteasome inhibitor, MG132, significantly increased the ubiquitination, but had no effect on the amounts of phosphorylated and unmodified forms. The level of ubiquitinated *IRAK-1KD* decreased markedly after transfection of IRAK-4. The decrease was again dependent on the IRAK-4 activity because the overexpression of *IRAK-4KD* did not show

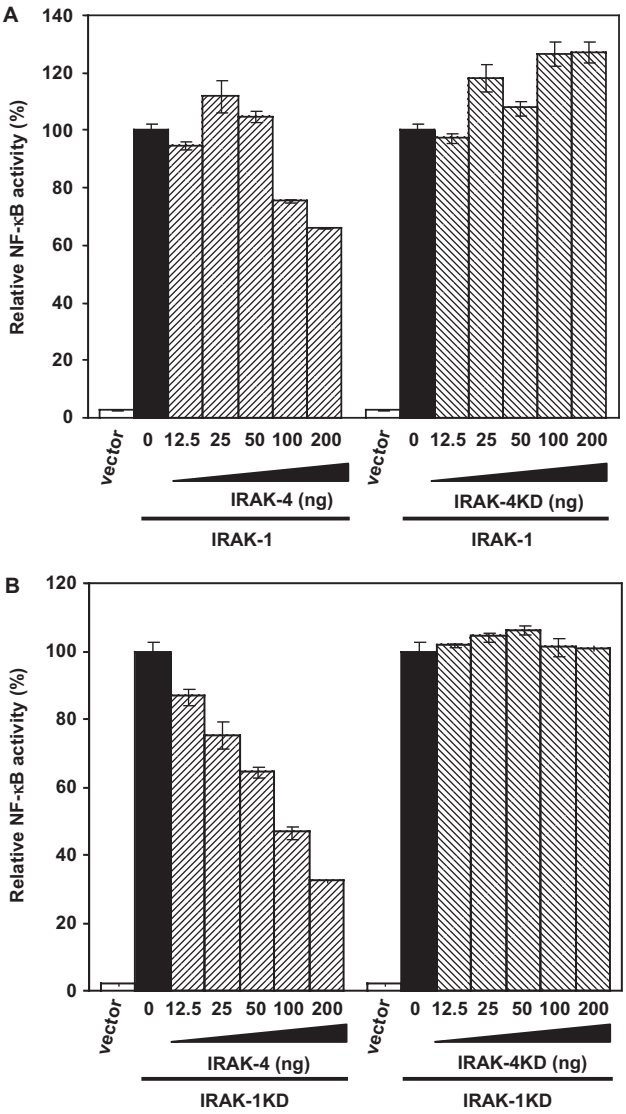

Fig. 3. **Inhibition of IRAK-1-induced NF-κB activation by IRAK-4 and *IRAK-4KD*.** HEK293T cells were transfected with 50 ng of the NF-κB reporter luciferase gene plasmid and 10 ng of the sea pansy luciferase gene plasmid, along with 20 ng of IRAK-1 (A) or *IRAK-1KD* (B). The indicated amounts of IRAK-4 or *IRAK-4KD* were also transfected. The total amount of transfected DNA was kept constant by supplementation with control vector. After 24 h, cell extracts were prepared and the normalized values of NF-κB-dependent expression were determined. The values are shown as percentages of those observed in the absence of IRAK-4 or *IRAK-4KD*. The results from three separate experiments, each of which was performed in duplicate, are shown as mean ± SD.

the similar effect (Fig. 4A, upper panel). MG-132 only partially inhibited the IRAK-4-induced decrease of the ubiquitinated *IRAK-1KD*. Therefore, overexpression of IRAK-4 in 293T cells is considered to decrease both the ubiquitinated and unmodified forms of *IRAK-1KD* through MG132-insensitive mechanisms.

Overexpression of TLR4, along with MD-2, in the HEK293T cells led to increased NF-κB activity probably owing to the activation of endogenous IRAKs (Fig. 5A). The addition of LPS caused only a minor enhancement of

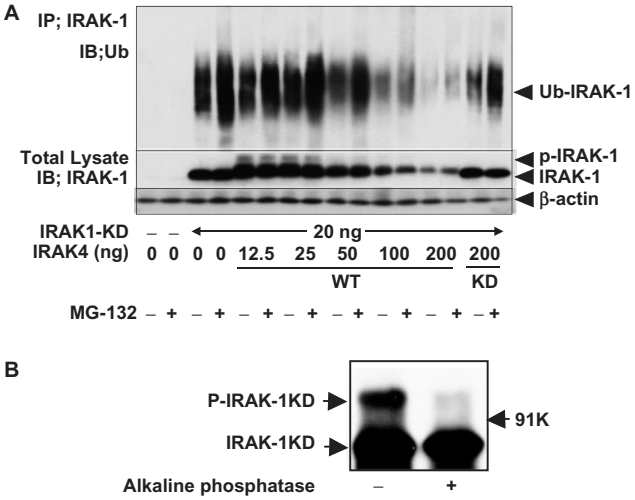

Fig. 4. **IRAK-4-dependent degradation of *IRAK-1KD*.** (A) HEK293T cells were transfected with *IRAK-1KD* (20 ng), together with increasing amounts of IRAK-4 or 200 ng of *IRAK-4KD*. After 24 h, the cells were treated with (+) or without (–) 30 μM MG-1132 for 1 h. Cell extracts were subjected to immunoprecipitation with anti-IRAK-1 antibody followed by western blot analysis with anti-ubiquitin antibody (top panel). The cell extracts were also subjected directly to western blot analysis with anti-IRAK-1 and anti-β-actin antibodies (middle and bottom panels, respectively). p-*IRAK-1KD*, phosphorylated *IRAK-1KD*; Ub-*IRAK-1KD*, ubiquitinated *IRAK-1KD*. (B) HEK293T cells were transfected with 20 ng each of *IRAK-1KD* and IRAK-4. After 24 h, the cells were lysed and mixed with anti-IRAK-1 antibody. The immune complex was incubated with (+) or without (–) alkaline phosphatase at 30°C for 15 min, and then washed three times before analysis by western blotting with anti-IRAK-1 antibody.

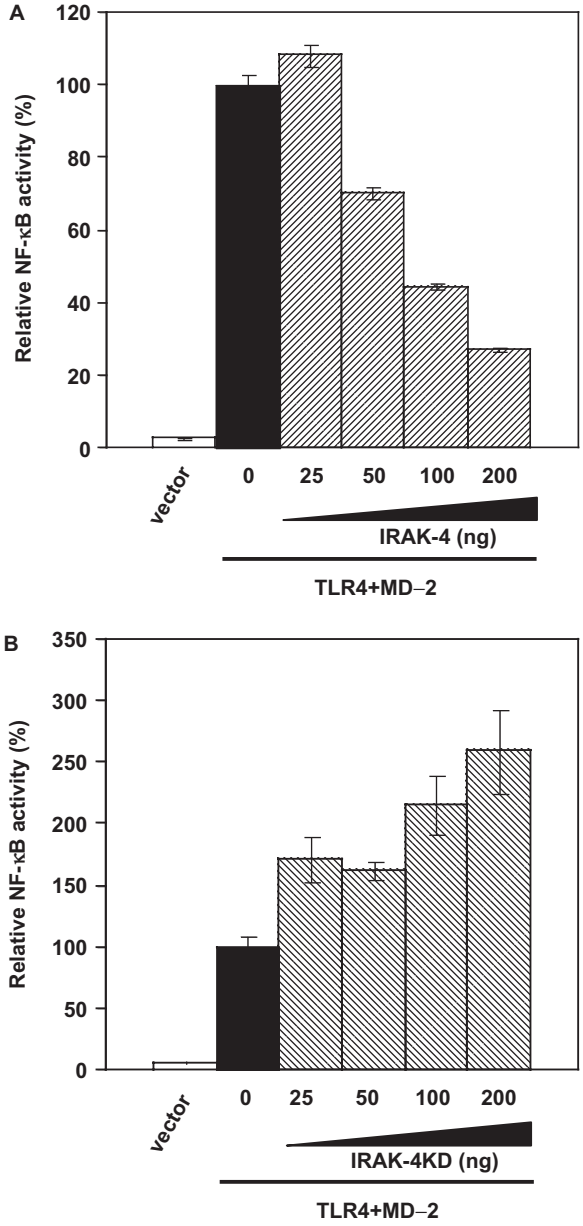

Fig. 5. **Inhibition of TLR4-induced NF-κB activation by IRAK-4.** HEK293T cells were transfected with 50 ng of the NF-κB reporter luciferase gene plasmid and 10 ng of the sea pansy luciferase gene plasmid, along with 50 ng of TLR4 and 10 ng of MD2. The indicated amounts of IRAK-4 (A) or *IRAK-4KD* (B) were also transfected. The total amount of transfected DNA was kept constant by supplementation with control vector. After 24 h, cell extracts were prepared and the normalized values of NF-κB -dependent expression were determined. The values are shown as the percentage of those observed in the absence of IRAK-4 or *IRAK-4KD*. The results from three separate experiments, each of which was performed in duplicate, are shown as mean ± SD.

the NF-κB activation in these cells (data not shown). The TLR4-dependent activation of κF-κB was inhibited by transfection of IRAK-4. By contrast, *IRAK-4KD* increased the TLR4-induced NF-κB activation (Fig. 5B), probably by acting as a dominant negative mutant against endogenous IRAK-4. In Fig. 6, a similar experiment was performed using HEK293 cells instead of the 293T cells. In these cells, overexpression of the receptors by itself did not result in the activation of NF-κB. However, LPS challenge to these cells induced a significant activation of NF-κB. The LPS-induced NF-κB activation was again inhibited by the IRAK-4 expression. These results are consistent with the theory that IRAK-4 possesses the ability to regulate negatively TLR signalling.

DISCUSSION

Li *et al*. (*21*) reported that overexpression of IRAK-4 increased BF-κB reporter activity. They also showed that a kinase-inactive mutant of IRAK-4 (*KK213AA*) inhibited the response to IL-1. In sharp contrast, Medvedev *et al*. (*22*) reported that neither wild-type nor mutant IRAK-4 caused NF- κB activation. They also reported that LPS- or IL-1-induced activation of NF-κB was down-regulated by wild-type IRAK-4. In agreement with the latter report, we did not observe any effect of IRAK-4 on the NF-κB activity of non-stimulated cells (data not shown). We also observed that wild-type IRAK-4 inhibited TLR4-induced NF-κB activation, whereas the kinase-deficient mutant increased NF-κB activation in HEK293T cells (Fig. 5). The present results, together with those of another group (*22*), indicate a potential role of IRAK-4 as a negative regulator of TLR signalling.

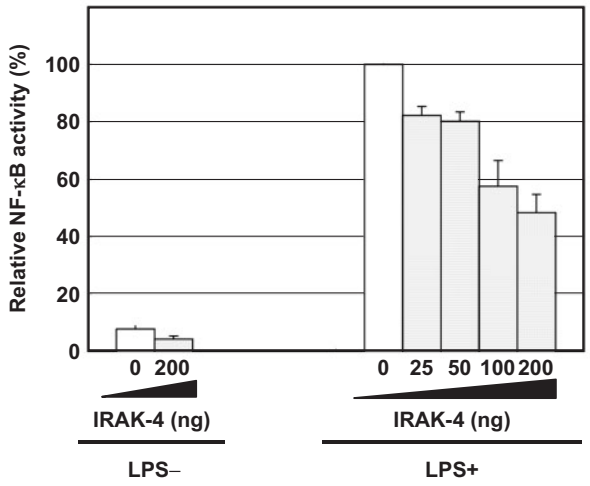

Fig. 6. **Inhibition of LPS-induced NF-κB activation by IRAK-4.** HEK293 cells were transfected with 50 ng of the NF-κB reporter luciferase gene plasmid and 10 ng of the sea pansy luciferase gene plasmid, along with 50 ng of TLR4 and 10 ng of MD2. The indicated amounts of IRAK-4 were also transfected. The total amount of transfected DNA was kept constant by supplementation with control vector. After 24 h, cells were stimulated with or without 100 ng/ml of LPS for 6 h. Cell extracts were prepared and the normalized values of NF-κB -dependent expression were determined. The values are shown as the percentage of those observed with LPS stimulation in the absence of IRAK-4. The results from three separate experiments, each of which was performed in duplicate, are shown as mean ± SD.

The inhibitory effect of IRAK-4 on NF-κB activation was surprising, because an indispensable role for IRAK-4 in TLR-signalling has been demonstrated in knockout mice (*7*), in patients with the IRAK-4 mutation (*22*) and using an alternative splicing variant of MyD88 which fails to recruit IRAK-4 (*13*). In agreement with the positive regulatory role of IRAK-4, we observed that the TLR-mediated activation of IKK was inhibited in ΔIRAK-4 cells (Fig. 2). We also observed that TLR-induced activation of Erk and p38 MAP kinase was severely impaired in the cells (data not shown). An intriguing finding was that TLR stimulation of ΔIRAK-4 cells showed no effect on the level of IRAK-1, in spite of the fact that the stimulation induced a marked and long-lasting decrease in the wild-type cells (Fig. 2). This finding, together with the results of the NF-κB reporter assay, suggested that IRAK-4-induced degradation of IRAK-1 is the mechanism responsible for the termination of TLR-mediated NF-κB activation. In this sense, IRAK-4 has both positive and negative regulatory roles in the TLR-signalling pathway. It is well documented that IRAK-4 is indispensable in MyD88-dependent signalling pathways. In one pathway, IRAK-4 enables IRAK-1 to activate NF-κB. In this pathway, the catalytic activity of IRAK-1 is not necessarily required (*9*, *10*). In another pathway, IRAK-1 leads to IFN-α production by directly phosphorylating IRF7 (*11*, *12*). IRAK-4-dependent degradation of IRAK-1 may be a termination mechanism in both signalling pathways.

Yamin and Miller (*23*) first reported that IL-1 stimulation induces the IRAK-1 phosphorylation and degradation in MRC-5 human lung fibroblast. They suggested that degradation of IRAK-1 leads to a shut-down of the IL-1 response and represents a negative feedback loop in the NF-κB pathway (*23*). Since the negative regulation by IRAK-4 functions only in IRAK-1-dependent pathways, the down-regulation may shift the sensibility of cells from the MyD88/IRAK-1-depedndent pathways to the independent ones. Thus, IRAK-4 may be necessary to terminate the first TLR-induced response and initiate the secondary autocrine responses to the secreted cytokines. The present result showing that degradation of IRAK-1 is not induced by *IRAK-4KD* indicates that the catalytic activity of IRAK-4 is necessary for this alternation of the signalling.

There have been conflicting reports on the involvement of IRAK-4 in TLR3-mediated cell events. It is proposed that TRIF is a sole adaptor for TLR3 (*19*, *3*). The adaptor TRIF associates with RIP1 (receptor-interacting protein-1), which leads to the activation of the IKK complex and NF-κB through the ubiquitin-dependent activation of TRAF6 (*24*, *25*). Alternatively, the recruitment of TRIF to TLR3 or TLR4 activates IRF-3 through the activation of IKKε, TBK1 and NAP1 (*26–28*). In both pathways, neither IRAK-1 nor IRAK-4 is required. A study with 293 cells deficient in IRAK1 supported the idea that IRAKs have no involvement in TLR3-mediated signalling (*29*). It has also been reported that TLR3- and TLR4-dependent induction of IFNs can effectively control viral infections, even in IRAK-4-deficient patients (*30*). By contrast, a study reported that TLR3-induced IFN-α production is impaired in these patients (*31*). Another study showed that poly(I-C) fails to generate IL-1, IL-6, TNF-α and nitrate in IRAK-4 knockout mice (*7*). It has also been reported that poly(I-C) does not induce IL-6 production in dendritic cells from IRAK-4-deficient patients (*32*). In the latter study, however, poly(I-C) was also reported to cause normal maturation of the cells (*32*). In the present study, we observed that poly(I-C)-induced IKK phosphorylation and IκB degradation were impaired, although still observable, in κIRAK-4 cells (Fig. 2B). We also observed in these cells that poly(I-C)-induced activation of Erk and p38 MAP kinase was attenuated (data not shown). Although the exact role of IRAK-1/4 in TLR3 signalling remains to be elucidated, the negative regulatory role of IRAK-4 may be operating in this system.

Although IRAK-4 is indispensable in the MyD88-dependent signalling pathway, the role of its kinase activity has remained elusive. It is reported that a kinase-inactive mutant of IRAK-4 has the same property as the wild-type IRAK-4 in IL-1-mediated signalling (*33*). The result suggests that catalytic activity of IRAK-4 is not necessarily required. This independence on the IRAK4 activity may be explained by the fact that the phosphorylation of IRAK-1 can be achieved by IRAK-1 itself (*33*, *34*). However, it has also been reported that the kinase activity of IRAK-4 is required for the optimal signal transduction of IL-1R (*34*). A recent study with 'knock-in' mice, which are generated by replacing the IRAK-4 gene with a mutant gene encoding *IRAK-4 KD*, has clearly shown that the catalytic activity of IRAK-4 is necessary for IL-1R-induced activation of NF-κB (*35*). Thus, the catalytic activity of IRAK-4 may be

indispensable for both the positive and negative regulation of TLR signalling pathway.

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