A novel splice variant of mouse interleukin-1-receptor-associated kinase-1 (IRAK-1) activates nuclear factor- κ B (NF- κ B) and c-Jun N-terminal kinase (JNK)

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Interleukin-1 (IL-1)-receptor-associated kinase (IRAK) is an indispensable signalling molecule for host-defence responses initiated by a variety of ligands that bind to members of the Toll/IL-1 receptor family. Here we report a novel splice variant of mouse IRAK-1, IRAK-1-S, which is generated by utilizing a new splicing acceptor site within exon 12. IRAK-1-S cDNA is shorter than the originally reported IRAK-1 (IRAK-1-W) cDNA by 271 nucleotides, and the subsequent frameshift causes a premature termination of translation after 23 amino acids, which are unique to the IRAK-1-S protein. To elucidate the physiological function of IRAK-1-S, we overexpressed it in 293T cells and studied the effects on the IL-1 signalling cascade. As it lacks the C-terminal region of IRAK-1-W that has been reported to contain the TRAF6 (tumour necrosis factor receptor-associated factor 6) binding domain, IRAK-1-S was unable to bind TRAF6 protein, which is a proposed downstream signalling molecule.

However, IRAK-1-S overexpressed in 293T cells induced constitutive activation of nuclear factor- κ B (NF- κ B) and c-Jun N-terminal kinase (JNK) independent of stimulation by IL-1, as did IRAK-1-W. To clarify the mechanism of NF- κ B activation by IRAK-1-S in the absence of binding to TRAF6, we demonstrated that IRAK-1-S binds to IRAK-1-W through its death domain; the findings suggested that overexpressed IRAK-1-S may bind endogenous IRAK-1-W and activate TRAF6 through IRAK-1-W. These results also indicate that this novel variant may play roles in the activation of NF- κ B and JNK by IL-1 and other ligands whose signal transduction is dependent on IRAK-1 under physiological conditions.

Key words: alternative splicing, death domain, IL-1-receptor-associated kinase (IRAK), IL-1 signalling, c-Jun N-terminal kinase (JNK), nuclear factor- κ B (NF- κ B).

INTRODUCTION

The Toll/interleukin-1 (IL-1) receptor (IL-1R) family contains a growing number of receptor molecules, such as IL-1R, IL-18R, ST2/T1 and the Toll-like receptor (TLR) family (TLR1–TLR10). These receptors are involved in a wide spectrum of physiological phenomena, including pro-inflammatory reactions, regulation of Th1/Th2 balance and innate immunity, when bound to a variety of ligands [1]. The receptors are thought to activate similar intracellular signalling cascades, including proximal adaptor proteins such as MyD88 and Mal, which are recruited to the receptor complex, and IL-1-receptor-associated kinase (IRAK), a serine/threonine kinase, which is in turn recruited to MyD88. IRAK is then phosphorylated and binds TRAF6 [tumour necrosis factor (TNF) receptor-associated factor 6], and this complex in turn activates TAK1 (transforming growth factor- β -activated kinase 1) and further signalling cascades that activate nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinases such as c-Jun N-terminal kinase (JNK) and p38 [1–4].

Among these signalling molecules, IRAK was shown to be indispensable by experiments using knock-out mice and mutant cells [5–8]. Furthermore, IRAK appeared to be a point of divergence in the signalling cascade towards either NF- κ B or JNK when cells were stimulated with IL-1 or lipopolysaccharide

[9,10]. The IRAK family is currently known to contain four related kinases: IRAK-1, IRAK-2, IRAK-M and the recently cloned IRAK-4 [11–15]. However, with the exception of IRAK-4 [15,16], the kinase activity of these molecules is thought to be non-essential for signal transduction [13], and phosphorylation of the IRAK protein after stimulation by IL-1 is thought to be due, at least partly, to autophosphorylation; the details of these mechanisms remain to be clarified. Moreover, the relationship and correspondence between the IRAK family and the Toll/IL-1R family are poorly understood and remain to be elucidated. The domain structure of IRAK-1 has also been studied. The N-terminal death domain of IRAK-1 is thought to be involved in binding to the MyD88 adaptor protein, the kinase domain is located in the central region of IRAK-1, and the C-terminal domain contains the TRAF6-binding domain [10,17–19]. In addition, a splicing variant of IRAK-1 (IRAK-1b) that lacks 30 amino acids at the C-terminal end of the kinase domain was reported recently by Jensen and Whitehead [20]. This variant had relatively prolonged stability, but its properties were similar to those reported originally for IRAK-1 [20].

In the course of studying the physiological functions of an orphan receptor, ST2/T1, which belongs to the IL-1R family [21], we cloned a novel splice variant of mouse IRAK-1, termed IRAK-1-S, which was more truncated than IRAK-1b. The

Abbreviations used: EMSA, electrophoretic mobility-shift assay; HA, haemagglutinin; IL-1, interleukin-1; IL-1R, IL-1 receptor; IRAK, IL-1-receptor-associated kinase; IRAK-1-W, mouse full-length IRAK-1 reported originally (aa 1–710); IRAK-1-S, novel splice variant of IRAK-1; IRAK-1-W- Δ N, N-terminally truncated IRAK-1-W (aa 34–710 of IRAK-1-W); JNK, c-Jun N-terminal kinase; NF- κ B, nuclear factor κ B; RT-PCR, reverse transcription-PCR; TAK, transforming growth factor- β -activated kinase; TLR, Toll-like receptor; TNF, tumour necrosis factor; TRAF6, TNF-receptor-associated factor 6.

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The novel nucleotide sequence data reported will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession numbers AB088370 (cDNA sequence covering the whole coding region of IRAK-1-S) and AB088390 (genomic sequence covering introns 11–12 of mouse IRAK-1).

IRAK-1 gene has been shown to produce a variety of isoforms; further investigation is necessary to achieve an understanding of the functional roles of this complicated signalling molecule in inflammation and host-defence systems. To elucidate the function of IRAK-1-S protein, we analysed the effects of overexpressing IRAK-1-S on the IL-1 signalling system. In the present paper, the sequence and structure of the novel splicing variant of IRAK-1 are revealed, and we also discuss our findings that the IRAK-1-S protein activates both NF- κ B and JNK, and that it forms a complex with the IRAK-1-W protein [full-length IRAK-1 reported originally (aa 1–710)].

EXPERIMENTAL

Cell culture and transfection

Human embryonic kidney 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml), and transfection was performed with the indicated cDNAs by a conventional calcium phosphate method. At 16 h after transfection the culture medium was changed to fresh medium, and on the following day the cells were harvested and submitted to subsequent analysis. When the cells were to be stimulated by IL-1, the culture medium was changed to Dulbecco's modified Eagle's medium with 0.5% (v/v) fetal calf serum at 16 h after transfection. Cells were further cultured 24 h later with human IL-1 α (10 ng/ml; a gift from Professor Tadashi Kasahara, Kyoritsu College of Pharmacy, Tokyo, Japan) or left untreated for the indicated period.

Reverse transcription-PCR (RT-PCR), and cloning of IRAK-1-S cDNA and a relevant genomic DNA fragment

The expression of mouse IRAK-1 isoforms was assessed using RNAs from various mouse tissues, BALB/c-3T3 mouse fibroblasts and EL-4 mouse thymoma-derived cells by RT-PCR as described previously [21], with primers F1 (5'-CGACTGGCTGTGGACACCGATAC-3') and R1 (5'-CCTGGGCTACTCTCACACTTGATTC-3'). The entire coding region of the truncated mouse IRAK-1 cDNA was amplified from the total RNA derived from EL-4 cells by RT-PCR with primers F2 (5'-GCCATGGCCGGGGGG-3') and R2 (5'-TCAGCTCTGGAA-TTCATCACTTTCTTCAGGTC-3'), which contain an initiation codon and a stop codon respectively (GenBank accession no. AF103876). The cDNA was then cloned and sequenced (DDBJ accession no. AB088370) with an ABI PRISM 310 sequencer (Applied Biosystems Inc., Foster City, CA, U.S.A.). A partial genomic DNA fragment that is relevant to the generation of IRAK-1-S was amplified from genomic DNA of EL-4 cells by PCR with primers F1 (5'-CGACTGGCTGTGGACACCGAT-AC-3') and R3 (5'-CTTCTGTCTGGGCTGGGTTGATGATG-ATCT-3'), and the PCR product was also cloned and sequenced (DDBJ accession no. AB088390).

Plasmids

The expression plasmids used in this study were constructed from a pCMV5-based plasmid by standard PCR and cloning techniques. cDNAs for IRAK-1-W (corresponding to aa 1–710), IRAK-1-W- Δ N (aa 34–710), IRAK-1-113 (aa 1–113) and IRAK-1-203 (aa 1–203) were cloned according to the nucleotide sequence in the database (GenBank accession no. AF103876). The pCMV5-FLAG- Δ TRAF6 plasmid was derived from mouse TRAF6 (aa 295–530). pCMV5-Myc-AKT-KD was derived from human AKT (K177A) and pCMV5-Myc-TAK1-KD was derived

from mouse TAK1 (K63A) (both designed to generate the dominant negative kinase). Mouse IL-1RI cDNA was cloned into pEF-BOS, as described previously [22].

In vitro kinase assay

For the detection of IRAK activity, 293T cells were transfected with pEF-BOS-IL-1R and pCMV5-Myc-IRAK-1-W or pCMV5-Myc-IRAK-1-S (see above), and then 2 days later the cells were incubated with or without IL-1 α for 15 min. After the incubation, cells were washed immediately with PBS and lysed in 1 ml of 50 mM Hepes, pH 7.4, 150 mM NaCl, 1% (w/v) Nonidet P40, 1 mM EDTA, 2 mM dithiothreitol, 20 mM β -glycerophosphate, 1 mM sodium orthovanadate, 5 mM *p*-nitrophenyl phosphate, 50 mM NaF and 1 tablet/50 ml CompleteTM Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, U.S.A.). Anti-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) was added to the lysate, and then precipitated with Protein A/G PLUS-agarose (Santa Cruz Biotechnology). The precipitated agarose beads were then washed four times with lysis buffer and suspended in kinase buffer, and the *in vitro* kinase assay was performed as described in the literature [23].

For the JNK assay, 293T cells were transfected with haemagglutinin (HA)-tagged JNK plasmid with or without other expression plasmids, as indicated. After 2 days, the cells were stimulated with IL-1 α for the indicated periods and then lysed in 1 ml of 20 mM Tris/HCl, pH 7.4, 0.5% (w/v) Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM β -glycerophosphate, 1 mM sodium orthovanadate, 10 mM NaF and Complete Protease Inhibitor Cocktail. Immunoprecipitation was performed with anti-HA antibody (Roche Applied Science), and the precipitated pellet was assayed for JNK activity, as described previously [24]. Phosphorylated proteins were separated by SDS/PAGE and analysed with a BAS 2000 system (Fuji Film, Tokyo, Japan).

Luciferase assay

The reporter plasmids pNF- κ B-Luc (0.1 μ g; Stratagene, La Jolla, CA, U.S.A.) and pRL-TK (0.01 μ g; Promega, Madison, WI, U.S.A.) were transfected with other expression plasmids, as indicated, into 293T cells cultured in a 35 mm dish. After 2 days the cells were treated or not with 10 ng/ml IL-1 α for 12 h, and then harvested. Cells were lysed with passive lysis buffer (Promega), and duplicate lysates were analysed for both firefly and *Renilla* luciferase activities using a dual-luciferase reporter assay kit (Promega). The activity of firefly luciferase under the control of NF- κ B promoter was normalized to the activity of thymidine kinase-promoter-driven *Renilla* luciferase. Each experiment was repeated three times, and the mean values of representative results are presented.

Electrophoretic mobility-shift assay (EMSA)

293T cells were transfected with the relevant plasmids followed by a 2 day cultivation; where indicated, the cells were treated with 10 ng/ml IL-1 α or TNF α for 15 min. Cells were washed with PBS, lysed in 10 mM Hepes/KOH, pH 7.8, 10 mM KCl, 0.1% (w/v) Nonidet P40, 0.1 mM EDTA and Complete Protease Inhibitor Cocktail, and then spun at 1400 *g* for 1 min at 4 °C. Each nuclear pellet was resuspended in 50 mM Hepes/KOH, pH 7.8, 420 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 2% (v/v) glycerol and Complete Protease Inhibitor Cocktail, and left on ice for 30 min with occasional vortexing. Mixtures were then spun at 12000 *g* for 15 min at 4 °C. Each supernatant (nuclear extract) was examined by EMSA, with the oligonucleotide 5'-

AGTTGAGGGGACTTTCCAGGC-3' used as a probe for NF- κ B binding, as described previously [24].

Immunoprecipitation and Western blotting

Tagged proteins transiently expressed in 293T cells were immunoprecipitated from cell lysates using anti-Myc antibody (Santa Cruz Biotechnology) or anti-FLAG antibody (Sigma, St. Louis, MO, U.S.A.) and Protein A/G PLUS-agarose (Santa Cruz Biotechnology), and were then separated by SDS/PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, U.S.A.). Western blotting analysis was performed with the indicated primary antibodies, with subsequent addition of horseradish peroxidase-conjugated anti-mouse immunoglobulin (Amersham Bioscience, Piscataway, NJ, U.S.A.) as the second antibody, and finally processed using an ECL[®] Western blotting detection system (Amersham).

RESULTS

Expression of IRAK-1-S in cell lines and tissues

In the course of studying the expression of mouse IRAK-1 in cell lines by RT-PCR, we discovered a truncated PCR product (Figure 1). The expression profile of this possible variant of mouse IRAK-1 was analysed further with total RNAs prepared from mouse organs; as shown in Figure 1, a distinct smaller band was observed with each organ. However, the truncated form was expressed more abundantly in the thymus (lane 6), spleen (lane 8) and kidney (lane 9) compared with the intact form derived from IRAK-1-W.

Cloning of IRAK-1-S, cDNA sequence, partial genomic DNA sequence surrounding the truncated region, and structure of IRAK-1-S protein

With primers F2 and R2, which contain the initiation codon and stop codon respectively of mouse IRAK-1-W cDNA, a truncated form of cDNA was also amplified, then cloned and sequenced. The cDNA was designated IRAK-1-S, and its sequence revealed that it lacks 271 nucleotides corresponding to nucleotides 1716–1986 of IRAK-1-W cDNA (accession no. AF103876). Owing to a premature termination, the deduced polypeptide contains 537 amino acids: 514 amino acids that are identical to those of IRAK-1-W starting from the initiation codon, followed by a 24-amino-acid sequence as the C-terminal region that is unique to the IRAK-1-S protein (accession no. AB088370). To investigate the mechanism of truncation, we cloned and sequenced a part of a mouse genomic DNA fragment surrounding the deleted region of IRAK-1-S cDNA. Since the entire genomic DNA sequence

for mouse IRAK-1 has not been made available to date, putative exons for mouse IRAK-1 were postulated according to the exon–intron organization of human IRAK-1 (accession no. GI 1302657) compared with the partial mouse genomic sequence already obtained. As shown in Figures 2(A) and 2(B), the deleted sequence of the mouse IRAK-1-S cDNA starts at the beginning of exon 12 and stops in the middle of the exon 12 at the sequence 5'-AG-3' (S in Figure 2A). This finding strongly suggests that IRAK-1-S mRNA is generated as a result of alternative splicing that utilizes a novel splicing acceptor site in exon 12. Thus exon 12 has at least three splicing acceptor sites: one for IRAK-1-W, the second for formerly reported IRAK-1b (b in Figure 2A) [20], and the third for IRAK-1-S. All of the exon–intron boundaries conform to the consensus splicing donor–acceptor sites (GT–AG rule).

The expected domain structure of the IRAK-1-S protein is depicted in Figure 2(C). The region identical with IRAK-1-W ends just before the C-terminal end of the kinase domain; the subsequent 23-amino-acid peptide is unique to IRAK-1-S protein, and shows no identity with other reported peptides.

IRAK-1-S protein does not show kinase activity

To evaluate whether IRAK-1-S protein possesses kinase activity, 293T cells were transfected with an expression plasmid for Myc-tagged IRAK-1-W or IRAK-1-S, and 2 days later the cells were treated or not with IL-1 α for 10 min. Then an *in vitro* kinase assay was performed with immunoprecipitate pulled down with anti-Myc antibody. IRAK-1-W protein expressed in 293T cells was constitutively active, and phosphorylated myelin basic protein added as substrate even in the absence of IL-1 stimulation (Figure 3, middle panel). The addition of IL-1 α augmented the degree of phosphorylation of myelin basic protein. A similar result was observed with regard to the autophosphorylation of IRAK-1-W protein itself, but this activity was weaker than the phosphorylation of myelin basic protein (Figure 3, top panel). In contrast, overexpressed IRAK-1-S protein showed no kinase activity, even in the presence of IL-1 α (Figure 3, lanes S).

IRAK-1-S protein constitutively activates NF- κ B in 293T cells

The lack of both the C-terminal region and kinase activity implied that IRAK-1-S plays an inhibitory role in the IL-1 signalling cascade. The effects of IRAK-1-S on the activation of NF- κ B by IL-1 were studied using a luciferase reporter assay. IRAK-1-S protein overexpressed in 293T cells induced the constitutive activation of NF- κ B-dependent luciferase expression, independent of IL-1 stimulation, as did IRAK-1-W protein (Figure 4A). Because only a limited amount of IL-1R is expressed in 293T cells, the IL-1R expression vector was co-transfected with IRAK isoforms. Under these conditions, an artificial IRAK isoform, IRAK-1-W- Δ N, that lacks 33 amino acids at the N-terminal end of IRAK-1-W, induced slight activation of NF- κ B (Figure 4A), as reported by Vig et al. [25]. To investigate the region of IRAK-1 that is required for NF- κ B activation, the deletion mutants IRAK-1-203, lacking the entire kinase region, and IRAK-1-113, consisting of only the death domain, were examined. IRAK-1-203 showed a distinct ability to activate NF- κ B to a similar extent as both IRAK-1-W and IRAK-1-S, but IRAK-1-113 induced only trace activation of NF- κ B, and inhibited IL-1-dependent NF- κ B activation (Figure 4A).

When a dominant negative form of MyD88 (Δ MyD88) was co-expressed with IRAK-1-S in 293T cells, the constitutive activation of NF- κ B by IRAK-1-S was not attenuated, similar to

Figure 1 Expression of IRAK-1-S mRNA in cell lines and tissues

RT-PCR was performed with primers F1 and R1, located in putative exons 11 and 12 of IRAK-1. Each template cDNA was derived from 0.5 μ g of total RNA prepared from the indicated mouse cell lines or mouse tissues. W and S indicate the PCR products derived from IRAK-1-W and IRAK-1-S mRNAs respectively. Lane 1, BALB/c-3T3 cells; lane 2, EL-4 cells; lane 3, brain; lane 4, heart; lane 5, lung; lane 6, thymus; lane 7, liver; lane 8, spleen; lane 9, kidney; lane 10, testis.

Figure 4 NF- κ B activation by the overexpression of IRAK-1-S protein

293T cells were transfected with pCMV5-based plasmids containing cDNAs as indicated, pEF-BOS-IL-1R, pNF- κ B-Luc, and pRL-TK plasmids; then, 2 days after transfection, the cells were incubated with (■) or without (□) IL-1 α for 12 h. The cells were lysed and assayed for luciferase activity. The data were normalized to *Renilla* luciferase activity and are represented as the means of duplicate assays. Similar results were obtained in at least three independent experiments. (A) Cells were co-transfected with pEF-BOS-mouse IL-1R type I (IL-1R). W and S indicate the expression of IRAK-1-W and IRAK-1-S protein respectively. W- Δ N indicates data derived from cells transfected with pCMV5-Myc-IRAK-1-W- Δ , encoding IRAK-W protein lacking 33 amino acids at the N-terminal end. The expression of IRAK-1-113 or IRAK-1-203 is indicated as 113 and 203 respectively. (B) Δ TRAF6 indicates the expression of dominant negative TRAF6 protein in 293T cells. The amount of the expression plasmid for Δ TRAF6 used to transfect 293T cells in a 35 mm culture dish is indicated. (C) The indicated plasmids were transfected into 293T cells with or without pEF-BOS-IL-1R. Akt-KD and TAK1-KD indicate kinase-inactive mutants of Akt and TAK1 respectively. EV, empty vector.

binding activity for the oligonucleotide probe containing the NF- κ B binding site (Figure 5). As a control, TNF α induced marked NF- κ B activity in 293T cells, and the main component of the activated NF- κ B was p65/RelA, with a trace amount of p50, as revealed by supershift assay (Figure 5, EV, TNF α +). The profile of EMSA with IL-1 stimulation was essentially the same as that observed with TNF α (Figure 5, IL-1R, IL-1 +). When 293T cells were transfected with IRAK-1-W, IL-1-independent NF- κ B DNA-binding activity was observed; however, the major components of NF- κ B were p65/RelA and c-Rel, with a small amount of p50. The overexpression of IRAK-1-S, IRAK-1-203 or IRAK-1-113 protein in 293T cells had a similar effect

Figure 5 Activation of NF- κ B subunits by IRAK-1-S

293T cells were transfected with expression plasmids as indicated. After 2 days, cells were left untreated (—) or stimulated (+) with TNF α (10 ng/ml) or IL-1 α (10 ng/ml) for 15 min, then nuclear extracts were prepared from the cells. Antibodies against NF- κ B subunits (50, p50; A, RelA/p65; C, c-Rel) were incubated with the nuclear extracts (4 μ g of protein per assay), and after the binding reaction of these nuclear extracts with a 32 P-labelled NF- κ B oligonucleotide probe, PAGE for EMSA was performed. The gel was dried and the probe bound to NF- κ B was analysed with a BAS 2000 system. EV, empty vector.

on the profile of activated NF- κ B as IRAK-1-W, but the induction of DNA-binding activity was small with IRAK-1-113 (Figure 5).

IRAK-1-S activates JNK in the absence of stimulation by IL-1

We then analysed activation of JNK as another IL-1-dependent effect by *in vitro* kinase assay. As shown in Figure 6, JNK was activated in an IL-1-dependent manner in 293T cells transfected with IL-1R. When IRAK-1-W was co-transfected with IL-1R, the constitutive activation of JNK with c-Jun protein as a substrate and phosphorylation of IRAK-1-W that was co-immunoprecipitated with HA-tagged JNK were observed without IL-1 stimulation. After stimulation with IL-1 for 15 min, JNK activity towards c-Jun protein was enhanced, but the band of phosphorylated IRAK-1-W disappeared. After 30 min of IL-1 stimulation, the band of phosphorylated IRAK-1-W became visible again, and JNK activity towards c-Jun protein returned to the level observed before IL-1 stimulation. When IRAK-1-S was

Figure 6 IRAK-1-S activates JNK

293T cells were transfected with expression plasmid encoding HA-tagged JNK with or without the expression plasmids for IL-1R type I, IRAK-1-W or IRAK-1-S. At 2 days after transfection, the cells were treated with or without IL-1 α (10 ng/ml) for the indicated periods, then harvested and immunoprecipitated with anti-HA antibody. An *in vitro* kinase assay was performed with the immunoprecipitates, and the phosphorylation of c-Jun added as the exogenous substrate (indicated by the arrow) was detected using a BAS 2000 system after SDS/PAGE. The position of phosphorylated IRAK-1-W is indicated by the arrowhead.

Figure 7 IRAK-1-S does not bind TRAF6, but does bind IRAK-1-W

(A) 293T cells were transfected with pCMV5-FLAG-TRAF6 and a pCMV5-Myc plasmid containing IRAK-1-W (W), IRAK-1-S (S) or IRAK-1-W- ΔN (ΔN) cDNA, and immunoprecipitation (IP) and immunoblotting (IB) were performed with anti-FLAG or anti-Myc antibodies (indicated by FLAG and myc respectively). (B) 293T cells were transfected with pCMV5-FLAG-IRAK-1-W plus a pCMV5-Myc plasmid encoding IRAK-1-W (W), IRAK-1-S (S), IRAK-1-W- ΔN (ΔN), IRAK-1-113 (113) or IRAK-1-203 (203). Immunoprecipitation and immunoblotting were performed as in (A), and the bands corresponding to IRAK-1 isoforms are indicated as W, S, ΔN , 203 and 113 (arrows).

co-transfected with IL-1R, constitutive activation of JNK was also detected, but IL-1-dependent enhancement of JNK activity was less obvious compared with the experiment with IRAK-1-W; furthermore, no phosphorylated band corresponding to IRAK-1-S protein was detected.

Without co-transfection of IL-1R and IL-1 stimulation, IRAK-1-W induced JNK activity by itself, and a phosphorylated band of IRAK-1-W was also detected. IRAK-1-S alone was also able to activate JNK activity without stimulation (Figure 6).

IRAK-1-S binds to IRAK-1-W protein, but not to TRAF6 protein

The C-terminal region of IRAK-1-W protein has been reported to bear a TRAF6-binding site [19]. As it lacks this region, IRAK-1-S was expected not to bind to TRAF6 protein. To address this issue, immunoprecipitation and Western blotting was performed with FLAG-tagged TRAF6 protein and Myc-tagged IRAK-1 isoforms expressed in 293T cells. As shown in Figure 7(A), IRAK-1-W protein showed ability to bind TRAF6, but the binding was not IL-1-dependent in this system (Figure 7A). IRAK-1-W- ΔN protein also showed slight binding activity, but IRAK-1-S could not bind TRAF6 protein, as expected.

As it lacks the ability to bind TRAF6, and has no involvement in the phosphoinositide 3-kinase/Akt pathway (as shown in Figure 4), it is possible that IRAK-1-S activates NF- κ B and JNK by interaction with other signalling molecules. Thus we studied the ability of IRAK-1-S to bind IRAK-1-W. As reported previously, IRAK-1 protein formed a homodimer [13]: FLAG-tagged IRAK-1-W co-immunoprecipitated with Myc-tagged IRAK-1-W. IRAK-1-S protein also showed binding activity with IRAK-1-W protein (Figure 7B). On the other hand, IRAK-1-W- ΔN , which lacks a portion of the death domain, was unable to bind IRAK-1-W. We next studied the importance of the death domain in binding IRAK-1-W using truncated IRAK-1 variants. IRAK-1-203 and IRAK-1-113 were both able to bind IRAK-1-W (Figure 7B), which suggests that the death domain of IRAK-1 is important and sufficient for the binding of IRAK-1 proteins.

DISCUSSION

In this study, we cloned a novel splicing variant of IRAK-1, IRAK-1-S, and demonstrated its ability to activate NF- κ B and JNK as representative outputs of the IL-1 signalling pathway. Previously, Jensen and Whitehead [20] reported another splicing variant of IRAK-1, IRAK-1b, that lacks the first 90 nucleotides of exon 12 of the human IRAK-1 gene, such that the resulting IRAK-1b protein has an in-frame deletion of 30 amino acids at the C-terminal end of the kinase domain. On the basis of sequence analysis, IRAK-1-S mRNA appears to be generated by a similar alternative splicing mechanism to that of IRAK-1b; however, the first 271 nucleotides in putative exon 12 of the mouse IRAK-1 gene are missing from the IRAK-1-S cDNA, and the subsequent frame shift and premature termination generate a highly truncated IRAK-1-S protein (Figure 2). The mechanism of the convergence of at least three splicing acceptor sites in exon 12 of the IRAK-1 gene is unclear, and it remains to be clarified whether or not alternative splicing sites in the IRAK-1 gene are limited to exon 12. We attempted to clone the counterpart of human IRAK-1-S cDNA, but only a truncated cDNA lacking the whole of exon 12 was cloned (K. Yanagisawa, unpublished work).

The expression of IRAK-1-S mRNA was almost ubiquitous in mouse organs when studied by RT-PCR (Figure 1), but the amount of IRAK-1-S protein expressed remains unclear. A specific antibody for IRAK-1-S protein is not available to date; therefore we constructed an expression plasmid for Myc-tagged IRAK-1-S and studied the function of the overexpressed protein.

As expected from the report revealing that IRAK-1b is kinase-inactive [20] and the fact that a larger region is deleted in IRAK-1-S than in IRAK-1b, the IRAK-1-S protein was also shown to be kinase-inactive and not phosphorylated following IL-1 stimulation, unlike the IRAK-1-W protein (Figure 3). Whereas IRAK-1b retains a TRAF6-binding site in its C-terminal region because of its in-frame truncation, IRAK-1-S lacks the entire C-terminal region following the kinase domain of the IRAK-1-W protein;

hence IRAK-1-S protein does not bind to TRAF6 (Figure 7A). This finding led us to propose that IRAK-1-S would act as a dominant negative molecule on IL-1 signalling pathways; however, by luciferase assay and EMSA, IRAK-1-S was shown to activate NF- κ B in the absence of stimulation by IL-1 when overexpressed in 293T cells (Figures 4 and 5). The relationship between the function and domain structure of IRAK-1 protein has been studied in detail using artificial variants [10,17,18], and a construct designated IRAK-NK(1–532) that is highly similar to IRAK-1-S was reported to suppress the activation of NF- κ B in HeLa cells [17]. On the other hand, IRAK-1-S was shown to be a potent activator of NF- κ B when expressed in 293T cells. This discrepancy may be due to differences between cell lines. Further studies with deletion mutants revealed that the death domain of IRAK-1 (IRAK-1-113) was able to induce relatively weak activation of NF- κ B when overexpressed; however, the death domain plus the unique domain between the death domain and the kinase domain (IRAK-1-203) was more efficient at activating NF- κ B (Figures 4A and 5), which suggests that this unique domain also plays an important role in NF- κ B activation.

Even though it lacks the ability to bind TRAF6, IRAK-1-S appeared to induce TRAF6-dependent activation of NF- κ B, based on the study using the dominant negative TRAF6 protein (Δ TRAF6) (Figure 4B). Therefore we studied binding activity between IRAK-1-S and IRAK-1-W. IRAK-1-W protein was revealed to form a homodimer, as reported by Wesche et al. [13], but IRAK-1-W- Δ N did not bind IRAK-1-W, as shown by immunoprecipitation and Western blotting (Figure 7B). IRAK-1-S, IRAK-1-203 and IRAK-1-113, which retain the entire death domain, showed binding activity with IRAK-1-W protein (Figure 7B). Thus we conclude that the death domain is responsible for the dimerization of IRAK-1 isoforms. Taken together, these studies suggest that dimerization of IRAK-1-S with endogenous IRAK-1-W in 293T cells accounts for the activation of NF- κ B by IRAK-1-S, through the activation of TRAF6, which is recruited to endogenous IRAK-1-W. Mutant cells that lack the IRAK-1 gene will provide a good tool for investigation of the physiological roles of the IRAK-1-S protein, but these remain to be identified in future studies.

The activation of NF- κ B by IRAK-1-S was also examined by EMSA (Figure 5). The subunits of NF- κ B activated by TNF α and IL-1 α were mainly RelA/p65 and p52, whereas c-Rel was also highly activated (in addition to RelA/p65 and p52) by the overexpression of IRAK-1-W or IRAK-1-S. This discrepancy suggests that stimulation by IL-1 may activate other signalling pathways that inhibit the activation of c-Rel independent of IRAK-1, and it would be useful to investigate further the mechanisms for the activation of discrete subunits of NF- κ B by IRAK-1 or IL-1.

We also studied the activation of JNK by IRAK-1 proteins. IRAK-1-W overexpressed in 293T cells induced the constitutive activation of JNK and the phosphorylation of IRAK-1-W protein that co-immunoprecipitated with JNK protein. When the cells were stimulated with IL-1, JNK activity was enhanced transiently after 15 min, and phosphorylated IRAK-1-W disappeared. After 30 min, however, JNK activity diminished to a level similar to that observed before stimulation, and the band of phosphorylated IRAK-1-W was once again detected (Figure 6). In the case of 293T cells co-transfected with JNK and IRAK-1-S, IL-1-independent JNK activation was observed, but no phosphorylated IRAK-1-S protein was detected. Transient degradation of IRAK-1 protein after its phosphorylation and subsequent ubiquitination following IL-1 stimulation was reported by Yamin and Miller [27], which would explain the

disappearance of the band of phosphorylated IRAK-1-W after IL-1 stimulation. However, this effect of IL-1 also suggests that the mechanism by which JNK is activated by the overexpression of IRAK-1-W protein is not identical with that brought about by IL-1, because stimulation by IL-1 further enhanced the activity of JNK induced by IRAK-1-W (Figure 6). Moreover, at least a portion of the IRAK-1-W protein was shown to form a complex with JNK protein, as these proteins were co-immunoprecipitated (Figure 6). We attempted to detect co-immunoprecipitation of IRAK-1-W protein with JNK by immunoprecipitation and Western blotting, but have failed thus far, probably due to the limited amount of IRAK-1-W that binds JNK. It also remains unclear why phosphorylated IRAK-1-S was not detected, i.e. it remains to be shown whether or not IRAK-1-S binds JNK, or whether IRAK-1-S perhaps is not phosphorylated under these circumstances. It would also be intriguing to identify whether or not the responsive kinase for the phosphorylation of IRAK-1-W is IRAK-1-W itself or JNK.

The function of IRAK-1-S protein remains unclear, but its binding activity indicates that it forms a complex with IRAK-1 proteins under physiological conditions. Once IRAK-1-W protein binds IRAK-1-S, the complex may bind only one TRAF6 molecule; this process would attenuate IL-1- and other IRAK-dependent signalling cascades. To elucidate the roles of IRAK-1-S in signalling systems other than IL-1-dependent signal transduction, it would be useful to investigate whether or not IRAK-1-S protein is involved in the signalling cascade initiated by TLR and ligands such as lipopolysaccharide.

The signal transduction systems that include the IRAK proteins are activated by a variety of ligands, such as IL-1, IL-18 and lipopolysaccharide. They are thought to utilize similar signalling molecules, including IRAK, but they appear to achieve highly diverse effects as regards host-defence responses. Following the report by Jensen and Whitehead [20], our finding of a novel splice variant of IRAK-1 has provided more evidence of the diversity of signalling through IRAK proteins, and this variant may play important roles in generating such manifold responses.

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REFERENCES

- O'Neill, L. (2000) The Toll/interleukin-1 receptor domain: a molecular switch for inflammation and host defence. *Biochem. Soc. Trans.* **28**, 557–566
- Akira, S., Takeda, K. and Kaisho, T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* **2**, 675–680
- Fitzgerald, K. A., Palsson-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Mansell, A. S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. T. et al. (2001) Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature (London)* **413**, 78–83
- Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z. and Matsumoto, K. (1999) The kinase TAK1 can activate the NIK-I κ B as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature (London)* **398**, 252–256
- Kanakaraj, P., Schafer, P. H., Cavender, D. E., Wu, Y., Ngo, K., Grealish, P. F., Wadsworth, S. A., Peterson, P. A., Siekierka, J. J., Harris, C. A. and Fung-Leung, W. (1998) Interleukin (IL)-1 receptor-associated kinase (IRAK) requirement for optimal induction of multiple IL-1 signaling pathways and IL-6 production. *J. Exp. Med.* **187**, 2073–2079
- Li, X., Commune, M., Burns, C., Vithalani, K., Cao, Z. and Stark, G. R. (1999) Mutant cells that do not respond to interleukin-1 (IL-1) reveal a novel role for IL-1 receptor-associated kinase. *Mol. Cell. Biol.* **19**, 4643–4652
- Thomas, J. A., Allen, J. L., Tsen, M., Dubnicoff, T., Danao, J., Liao, X. C., Cao, Z. and Wasserman, S. A. (1999) Impaired cytokine signaling in mice lacking the IL-1 receptor-associated kinase. *J. Immunol.* **163**, 978–984

- 8 Swantek, J. L., Tsen, M. F., Cobb, M. H. and Thomas, J. A. (2000) IL-1 receptor-associated kinase modulates host responsiveness to endotoxin. *J. Immunol.* **164**, 4301–4306
- 9 Muzio, M., Natoli, G., Sacconi, S., Leviero, M. and Mantovani, A. (1998) The human Toll signaling pathway: divergence of nuclear factor κ B and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J. Exp. Med.* **187**, 2097–2101
- 10 Li, X., Commane, M., Jiang, Z. and Stark, G. R. (2001) IL-1-induced NF κ B and c-Jun N-terminal kinase (JNK) activation diverge at IL-1 receptor-associated kinase (IRAK). *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4461–4465
- 11 Cao, Z., Henzel, W. J. and Gao, X. (1996) IRAK: a kinase associated with the interleukin-1 receptor. *Science* **271**, 1128–1131
- 12 Muzio, M., Ni, J., Feng, P. and Dixit, V. M. (1997) IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* **278**, 1612–1615
- 13 Wesche, H., Gao, X., Li, X., Kirschning, C. J., Stark, G. R. and Cao, Z. (1999) IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase (IRAK) family. *J. Biol. Chem.* **274**, 19403–19410
- 14 Suzuki, N., Suzuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S. et al. (2002) Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature (London)* **416**, 750–754
- 15 Li, S., Strelow, A., Fontana, E. J. and Wesche, H. (2002) IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 5567–5572
- 16 Knop, J. and Martin, M. U. (1999) Effects of IL-1 receptor-associated kinase (IRAK) expression on IL-1 signaling are independent of its kinase activity. *FEBS Lett.* **448**, 81–85
- 17 Cooke, E.-L., Uings, I. J., Xia, C. L., Woo, P. and Ray, K. P. (2001) Functional analysis of the interleukin-1-receptor-associated kinase (IRAK-1) in interleukin-1 β -stimulated nuclear factor κ B (NF- κ B) pathway activation: IRAK-1 associates with the NF- κ B essential modulator (NEMO) upon receptor stimulation. *Biochem. J.* **359**, 403–410
- 18 Qian, Y., Commane, M., Ninomiya-Tsuji, J., Matsumoto, K. and Li, X. (2001) IRAK-mediated translocation of TRAF6 and TAB2 in the interleukin-1-induced activation of NF κ B. *J. Biol. Chem.* **276**, 41661–41667
- 19 Darnay, B. G., Ni, J., Moore, P. A. and Aggarwal, B. B. (1999) Activation of NF- κ B by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF- κ B-inducing kinase; identification of a novel TRAF6 interaction motif. *J. Biol. Chem.* **274**, 7724–7731
- 20 Jensen, L. E. and Whitehead, A. S. (2001) IRAK1b, a novel alternative splice variant of interleukin-1 receptor-associated kinase (IRAK), mediates interleukin-1 signaling and has prolonged stability. *J. Biol. Chem.* **276**, 29037–29044
- 21 Li, H., Tago, K., Io, K., Kuroiwa, K., Arai, T., Iwahana, H., Tominaga, S. and Yanagisawa, K. (2000) The cloning and nucleotide sequence of human ST2L cDNA. *Genomics* **67**, 284–290
- 22 Yanagisawa, K., Naito, Y., Kuroiwa, K., Arai, T., Furukawa, Y., Tomizuka, H., Miura, Y., Kasahara, T., Tetsuka, T. and Tominaga, S. (1997) The expression of ST2 gene in helper T cells and the binding of ST2 protein to myeloma-derived RPMI8226 cells. *J. Biochem. (Tokyo)* **121**, 95–103
- 23 Croston, G. E., Cao, Z. and Goeddel, D. V. (1995) NF- κ B activation by interleukin-1 (IL-1) requires an IL-1 receptor-associated protein kinase activity. *J. Biol. Chem.* **270**, 16514–16517
- 24 Tago, K., Funakoshi, M., Mano, H., Yanagisawa, K., Hayakawa, M., Kuroiwa, K., Iwahana, H., Kasahara, T. and Tominaga, S. (2001) Presence of a genistein-responsive inhibitory mechanism on interleukin-1 α -induced NF- κ B activation. *Eur. J. Biochem.* **268**, 6526–6533
- 25 Vig, E., Green, M., Liu, Y., Donner, D. B., Mukaida, N., Goebel, M. G. and Harrington, M. A. (1999) Modulation of tumor necrosis factor and interleukin-1-dependent NF- κ B activity by mPLK/IRAK. *J. Biol. Chem.* **274**, 13077–13084
- 26 Madge, L. A. and Pober, J. S. (2000) A phosphatidylinositol 3-kinase/Akt pathway, activated by tumor necrosis factor or interleukin-1, inhibits apoptosis but does not activate NF κ B in human endothelial cells. *J. Biol. Chem.* **275**, 15458–15465
- 27 Yamin, T. and Miller, D. K. (1997) The interleukin-1 receptor-associated kinase is degraded by proteasomes following its phosphorylation. *J. Biol. Chem.* **272**, 21540–21547

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