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Identification and characterization of murine IRAK-2

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Abstract

Interleukin-1 receptor-associated kinases (IRAKs) are pivotal signaling elements of the Toll/IL-1 receptor (TIL) family, which play a role in innate immune responses by coordinating host defence mechanisms. Presently four different forms of human IRAK molecules are cloned (hu-IRAK-1, hu-IRAK-2, hu-IRAK-M, and hu-IRAK-4). In the murine system, only three genes have been identified so far, mouse Pelle-Like Kinase (mPLK), which corresponds to human IRAK-1, mu-IRAK-M, and mu-IRAK-4. Here we report the molecular cloning and characterization of murine IRAK-2 (mu-IRAK-2), a mouse homolog to human IRAK-2 (hu-IRAK-2). Murine and human IRAK-2 molecules show 67% sequence identity, they are ubiquitiously expressed, and both practically lack autophoshorylation kinase activity. The murine molecule reveals two remarkable differences to its human counterpart: it shows a C-terminal extension and it has no stimulatory effect on IL-1 induced NF-κB activation when compared to hu-IRAK-2, suggesting subtle functional differences in signaling by IRAK-2 in human and mouse cells.

Keywords: IRAK-1; IRAK-2; Mouse Pelle-Like Kinase; Toll/IL-1 receptor family; IL-1 signal transduction

The Toll/IL-1 receptor (TIR) family consists of two subfamilies of transmembrane proteins, which are involved in host defence mechanisms [1]. The molecules of the Toll-like receptor subfamily serve as pattern recognition receptors, recognizing a number of molecules derived from pathogens. Thus TLR2 and TLR4 mediate the cellular response to cell wall components derived from Gram-positive and Gram- negative bacteria [2,3], respectively, while TLR3 is activated by double-stranded viral RNA [4], TLR5 by bacterial flagellin [5], TLR6, in collaboration with TLR2, by MALP-2 [6], TLR7 by small anti-viral compounds [7], while TLR9 recognizes bacterial CpG DNA-motifs [8]. In contrast, the members of the IL-1 receptor subfamily are receptors for the cytokines of the IL-1 family, e.g., IL-1 and IL-18 which coordinate the beginning acute inflammation. The hallmark of all transmembrane members of the TIR family (with the exception of type II IL-1 receptor) is that all transmembrane molecules share a cytoplasmic domain, the TIR domain, which is absolutely required for signal transduction. In consequence, they share similar receptor-proximal signaling events.

The sequence of events initiating signal transduction is best studied in the prototypic IL-1 receptor complex. The first step is the binding of IL-1 to the type I IL-1 receptor (IL-1RI). Then the ligated receptor is recognized by the IL-1 receptor accessory protein (IL-1RAcP) and both form a heterodimeric transmembrane complex (for review see [9]). This results in the close spatial association of the individual TIR domains of receptor and co-receptor creating a scaffold for further protein interactions. With its own TIR domain the intracellular adapter molecule MyD88 [10,11] interacts with the TIR domains of IL-1RI and IL-1RAcP introducing a death domain into the active receptor complex. Via death domain protein interactions IRAK family members are recruited to MyD88 and thus to the active receptor complex. Subsequently, IRAK-1 becomes rapidly phosphorylated by IRAK-4 allowing further autophosphorylations, resulting in a pronounced alteration of the electrophoretic mobility of IRAK-1 in SDS–PAGE [12,13]. Hyperphosphorylated IRAK-1 leaves the receptor complex in order to interact with the downstream

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adapter TRAF6 [14]. The formation of the IRAK/TRAF6 complex initiates the assembly of a multiprotein signalosome, which then activates a number of downstream signaling pathways, finally resulting in the activation of transcription factors such as AP-1 and NF-κB (for review see [15]).

A serine/threonine-specific protein kinase activity was first identified in the IL-1 receptor complex in 1994 [16] and human IRAK-1 was cloned after co-purification with IL-1RI in 1996 [12]. In the same year, a murine homolog was identified termed mouse Pelle-Like Kinase (mPLK) [17] due to its strong homology to a kinase previously identified in insect cells. Today, three further human homologs, hu-IRAK-2, hu-IRAK-M, and recently hu-IRAK-4 and two mouse homologs, mu-IRAK-4 and mu-IRAK-M have been identified, cloned, and characterized [10,18–20].

Here we describe the identification and characterization of the mouse homolog of human IRAK-2. Murine (mu-IRAK-2) and human IRAK-2 show a sequence identity of 67%. They share the same overall domain structure and are highly conserved in their death and kinase domains. An obvious difference between the two molecules is that the C-terminus of mu-IRAK-2 is 35 amino acids longer than that of human IRAK-2. With respect to its function, mu-IRAK-2 behaves differently compared to human IRAK-2, in revealing no stimulatory effects on IL-1 induced NF-κB activation in overexpression systems in which hu-IRAK-2 shows a stimulatory capacity. Neither human nor murine IRAK-2 shows a detectable autophosphorylative capacity in in vitro kinase assays, due to an amino acid exchange in the substrate binding site [18].

Materials and methods

Reagents and cell culture. Recombinant human IL-1β was provided by Diana Boraschi (Dompe spA, L'Aquila, Italy). Anti-Flag monoclonal antibody M2 and M2-agarose (Sigma, Deisenhofen, Germany) were used for Western blotting and immunoprecipitation of Flag-epitope-tagged proteins. For transfection studies two adherent cell lines were used, the human embryonal kidney cell line HEK293 and the murine fibroblast cell line, IRAKminus which was generated in our laboratory from embryonic fibroblasts of an IRAK-1 knockout mouse [21], which were provided by W. Fung-Leung (R.W. Johnson Pharmaceutical Research Institute, San Diego, CA). Both cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The murine thymoma cell line EL-4 6.1 was propagated in RPMI medium supplemented with 5% fetal calf serum, 1 mM pyruvate, 2 mM L-glutamine, and non-essential amino acids.

Rapid amplification of cDNA ends (RACE) and cloning into an expression vector. The complete sequence of the mu-IRAK-2 gene was identified with a 3′ and 5′ RACE (RACE-Kit, Clontech, Palo Alto, CA) starting from the murine EST AI930103. The RACE reactions were performed with cDNA derived from RAW264.7 cells as template, following manufacturer's instructions. For the different reactions the following primers were used.

RACE	Primer
5′	mI2RACE-5′: 5′-CCCAGCCATGATGGGCCC TGTGTCTAGA-3′
5′-NESTED	mI2RACE-5′N: 5′-AGGGAACGTGGCTTCAC AGGCCGGACTT-3′
3′	mI2RACE-3′: 5′-GCCAGGGGCTGTGGCTAC TTCTAGAAGA-3′
3′-NESTED	mI2RACE-3′N: 5′-CCTCAAGGATGAACAGG AGAAAGTCCGG-3′

The complete cDNA of mu-IRAK-2 was obtained by PCR using cDNA from RAW264.7 cells as template. The expression vector of amino-terminally Flag-epitope-tagged mu-IRAK-2 was constructed by inserting the cDNA fragment in the mammalian expression vector pcDNA3-Flag (Invitrogen, San Diego, CA). To investigate the influence of the 35 amino acid enlarged C-terminus of mu-IRAK-2, compared to hu-IRAK-2, we generated the C-terminal truncated mu-IRAK-2 ΔC, where the last 35 amino acids of the C-terminus are deleted. Mu-IRAK-2 ΔC was cloned via PCR, with the full-length gene as template and the following primers:

5′-mI2/A	5′-CTATGCGGATCCGCTTGCTACATCTACC AGCTCCCGTCCT-3′
3′-mI2 ΔC	5′-GTTACTGAATTCTCATGTAGCGTTCTGG GGTGTCTGTGGGCC-3′

The expression vectors for hu-IRAK-1 and mPLK were provided by H. Wesche (Tularik, South San Francisco, CA).

RNA preparation and Northern blotting. Total RNA from cell lines and tissues for Northern blotting and preparation of first strand cDNA was isolated using the RNeasy Kit from Qiagen (Hilden, Germany). For Northern blotting, 10 μg RNA/lane were separated on a 1.2% formaldehyde agarose gel and blotted on to a nitrocellulose membrane [22]. Prehybridization and hybridization were carried out as described [22] at 42 °C. The probes were labeled with [α-^{32}P]dCTP by random oligonucleotide priming using a 350 bp PCR product of the gene as template.

Expression profiling. The pattern of mRNA expression of mu-IRAK-2 was investigated by PCR in a variety of tissues and cell lines. First strand cDNAs were used as templates with primers for the amplification of a 1500 bp fragment of the mu-IRAK-2 gene in a standard PCR (35 cycles of amplification). The PCR products were analyzed by agarose gel electrophoresis. The first strand cDNA was synthesized using 4 μg total RNA as template, 5 μM pd(N)$_6$ as random primer, 10 μM dNTPs and 5 U MMLV reverse transcriptase (RT) (Stratagene, La Jolla, CA). After denaturation of the RNA for 10 min at 65 °C, the complete reaction mix was incubated for 60 min at 37 °C and finally inactivated for 5 min at 95 °C.

Reporter gene assays. EL4 6.1 cells (5×10^6) were transfected by the DEAE/dextrane method [22] with 0.5 μg of 5× pNF-κB-luc (5× NF-κB consensus sequence in front of the luciferase gene, provided by W. Falk (Regensburg, Germany) and the indicated amounts of the mu-IRAK-2 expression plasmid. After 4 h, the cells were stimulated with IL-1β (10 ng/ml) for 18 h before harvest. Luciferase activity was determined and normalized on the protein content (Bradford assay) of the lysates.

Immunoprecitations and immunoblotting. For precipitation of Flag-epitope-tagged proteins, 2×10^6 HEK293 or IRAKminus cells were plated on 10 cm dishes and transfected on the following day by the calcium phosphate precipitation method as described [22]. The amount of coding plasmid was 10 μg for HEK293 cells and 25 μg for IRAKminus cells. Two days after transfection, the cells were washed in phosphate-buffered saline and lysed for 30 min in 1 ml lysis buffer (50 mM HEPES, pH 7.9, 250 mM NaCl, 20 mM β-glycerophosphate,

5 mM *p*-nitrophenyl phosphate, 1 mM EDTA, 1 mM sodium orthovanadate, 5 mM dithiothreitol, 1% Nonidet P-40, and protease inhibitors [complete (Roche, Mannheim, Germany)] at 4 °C. After centrifugation for 10 min at 15,000*g*, the supernatant was incubated with 30 μl M2-agarose beads (Sigma, Deisenhofen, Germany) for 2 h at 4° with gentle overhead rotation. The samples were split afterwards and 40% of the beads was used for the kinase assay (see below) and 60% for Western blotting. For Western blotting the beads were washed twice in lysis buffer and incubated in SDS sample buffer for 5 min at 95 °C. The proteins were separated by SDS–PAGE, transferred to a polyvinylidene difluoride membrane, and incubated with biotinylated anti-Flag M2 antibody (Sigma). The bands were detected with horseradish peroxidase coupled to streptavidine by an enhanced chemiluminescence reaction (Perbio, Helsingborg, Sweden).

In vitro kinase assays. After immunoprecipitation M2-agarose beads were washed twice with lysis buffer, once with kinase buffer (20 mM HEPES, pH 6.7, 15 mM NaCl, 5 mM $MgCl_2$, and 5 mM $MnCl_2$), and resuspended in 30 μl kinase buffer (with 1 μM ATP and 1 μCi [γ-^{32}P]ATP). The reaction mix was incubated under shaking for 20 min at 30 °C. The reaction was stopped by incubation in SDS sample buffer for 5 min at 95 °C. The samples were analyzed by SDS–PAGE. The gels were dried and exposed to an X-ray film (Kodak X-OMAT, Kodak, Rochester, USA).

Results

Identification of mu-IRAK-2

To identify the murine counterpart of human IRAK-2, we searched murine EST databases for similar sequences. A polypeptide sequence alignment of the murine EST AI930103 with hu-IRAK-2 revealed a significant homology. The full-length cDNA of mu-IRAK-2 was obtained via 3′ and 5′ RACE, starting from the identified EST. Mu-IRAK-2 is a protein of 622 amino acids with a calculated molecular mass of 69 kDa (Fig. 1). The overall sequence identity between mu-IRAK-2, hu-IRAK-2, and mPLK is between 67% and 26% (Table 1). The gene of mu-IRAK-2 is located on chromosome 6 and consists of 13 exons (Fig. 2), compared to the hu-IRAK-2 gene which consists reportedly of 12 exons and is located on chromosome 3. Mu-IRAK-2 has an extended C-terminus of 35 amino compared to hu-IRAK-2 (Fig. 1). The sequence of mu-IRAK-2 shows

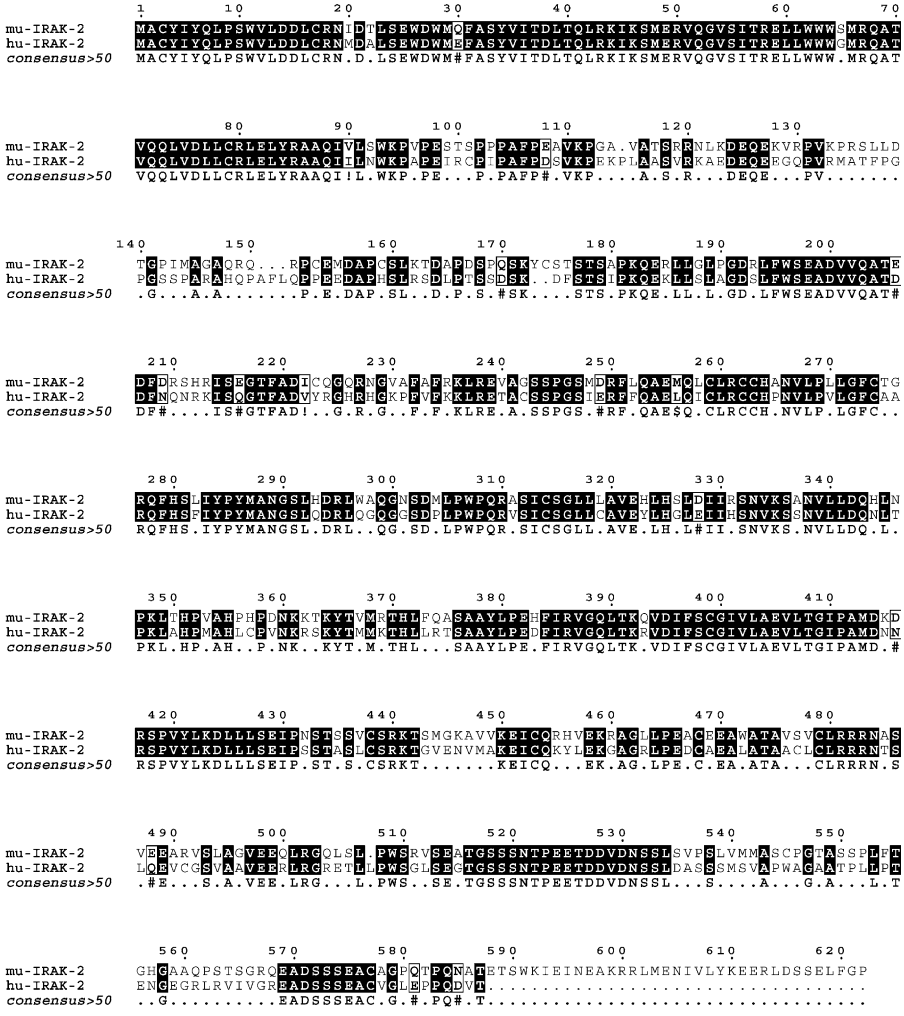

Fig. 1. Sequence alignment of murine and human IRAK-2. The protein sequences of hu-IRAK-2 (Accession No. AF026273) and mu-IRAK-2 (sequence was deposited under GenBank Accession No. AJ440756) were aligned using the multalin program [31]. Identical and similar residues are grouped in black and white boxes, respectively (used symbols: (·) different amino acids; (!) and (#) comparable amino acids).

Table 1
Sequence similarities between mu-IRAK-2, hu-IRAK-2, and mPLK were computed with BLAST

	mPLK	hu-IRAK-2
mu-IRAK-2	27%	67%
mPLK	—	26%

an N-terminal death domain, then an amino acid stretch rich in serine, threonine, and proline residues, followed by a conserved kinase domain with a mutation in the substrate binding site and a catalytic domain comparable to the other IRAK family members, terminated by the enlarged C-terminus compared to hu-IRAK-2.

Expression pattern of mu-IRAK-2

The expression pattern of mu-IRAK-2 was ascertained in several tissues and cell lines by Northern blot analysis using a ^{32}P-labeled specific cDNA probe (400 bp) and by RT-PCR of a 1500 bp fragment of the gene (data not shown). The Northern blot analysis revealed a single RNA species of about 4.4 kb. Mu-IRAK-2 was expressed in all tissues tested (Table 2), most prominently in brain, spleen, and liver. We also found mRNA expression in most of the investigated cell lines, such as IRAKminus, EL4 6.1, NIH3T3, and RAW264.7 and 70Z3 (Table 2) the cell line H1 did not express mRNA for mu-IRAK-2.

Analysis of NF-κB activation by mu-IRAK-2

It was shown that overexpression of the human molecules IRAK-1 (Fig. 3A), IRAK-2 (Fig. 3B), and IRAK-M in human cell lines increased IL-1-induced NF-κB activation in a dose-dependent manner [12,10,18]. This was also reported for mPLK [23], a result reproduced by us in this study (Fig. 3C). To investigate the influence of mu-IRAK-2 on IL-1 induced NF-κB activation, we transiently transfected EL-4 6.1 cells with increasing amounts of plasmid coding for mu-IRAK-2 in combination with an NF-κB-dependent luciferase reporter gene construct (Fig. 3E). In contrast to mPLK, which increases IL-1-induced NF-κB activation in a dose-dependent fashion, mu-IRAK-2 had no effect on IL-1 dependent NF-κB activation. To investigate, whether the C-terminal extension of 35 amino acids of mu-IRAK-2 (compared to the human IRAK-2) is responsible for the loss of the stimulatory effect on the IL-1 induced NF-κB activation, we constructed mu-IRAK-2 ΔC, where we deleted 35 amino acids of the C-terminus. Like mu-IRAK-2, mu-IRAK-2 ΔC showed no dose-dependent stimulatory effect on IL-1 induced NF-κB activation (Fig. 3D).

Autophosphorylation of mu-IRAK-2

Human IRAK-1 and murine mPLK both show a strong autophosphorylation in in vitro kinase assays [12,17]. In contrast, hu-IRAK-2 was reported to possess no autophosphorylative capacity [18]. To compare the in vitro kinase activities of hu-IRAK-1, mPLK, mu-IRAK-2, and mu-IRAK-2 ΔC we transiently transfected HEK293 cells with plasmids coding for the different Flag-epitope-tagged IRAK forms. IRAK proteins were immunoprecipitated with anti-Flag M2-agarose beads and subjected to an in vitro kinase assay. As it was published that the human forms of IRAK-1, IRAK-2, and IRAK-M can form heterodimers via their death domains and thus can be co-immunoprecipitated, we performed this set of experiments in HEK293 cells which possess endogenous hu-IRAK-1 and in IRAKminus cells, which lack endogenous murine IRAK-1, i.e., mPLK. The results obtained were identical for both cell lines. As expected, human IRAK-1 and mPLK showed

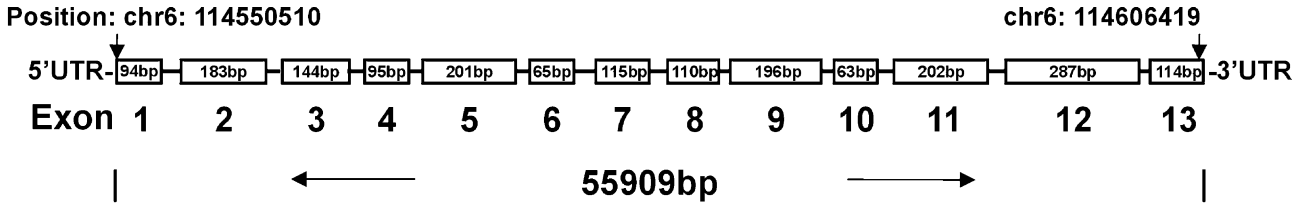

Fig. 2. Organization of the murine IRAK-2 gene. Data were generated with the UCSC Browser [32].

Table 2
Expression pattern of IRAK-2

Tissues:	Heart	Brain	Spleen	Liver	Kidney	Thymus
	+	++	++	++	+	+
Cell lines:	H1 (keratinocyte)	EL 4 6.1 (T-cell)	IRAKminus (embryonic fibroblast)	NIH 3T3 (embryonic fibroblast)	RAW 264.7 (monocyte/ macrophage)	70 Z 3 (pre B-cell)
	–	+	+	+	+	+

Data were collected using Northern blotting and RT-PCR. (–) No expression, (+) weak expression, and (++) strong expression.

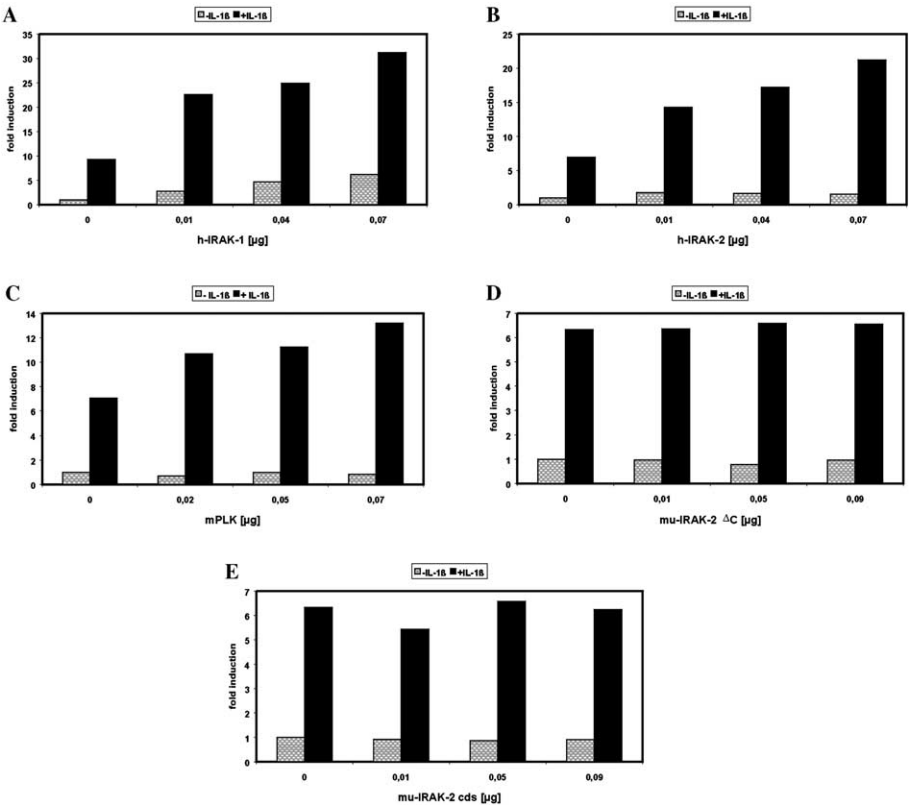

Fig. 3. Activation of NF-κB by different IRAK-forms. EL4 6.1 cells were transfected with a NF-κB-dependent luciferase reporter gene construct and the indicated amounts of expression plasmid for hu-IRAK-1 (A), hu-IRAK-2 (B), mPLK (C), mu-IRAK-2 ΔC (D), and mu-IRAK-2 (E). Four hours after transfection, cells were stimulated with IL-1β (10 ng/ml) for 18 h. Following, luciferase activities were determined and normalized on basis of the protein content. The *y*-axis represents the normalized fold of luciferase activity induction relative to cells transfected with the empty vector. All experiments were done three times with similar results.

heavy autophosphorylation, indicated by the broad and strong [^{32}P]-labeled bands, both molecules showing a pronounced shift after autophosphorylation (Fig. 4: lanes 1 and 2). In our hands, hu-IRAK-1 always showed a stronger overall phosphorylation compared to mPLK in in vitro kinase assay if the same amounts of proteins were applied, as controlled by the Western blot analysis. In contrast to hu-IRAK-1 and mPLK, there was no detectable autophosphorylation for mu-IRAK-2 ΔC and mu-IRAK-2 (Fig. 4: lanes 3 and 4). This result is in accordance with the data reported for hu-IRAK-2 [18].

Discussion

Interleukin-1 receptor-associated kinases (IRAKs) are signal transduction elements exclusively used by the TIR family. TIRs are crucial mediators of host defence, which function as receptors for structures and molecules derived from pathogens as well as for endogenous pro-inflammatory cytokines (for review see [15]). We first described an IL-1 receptor-associated protein kinase activity in 1994 [16], today four different human IRAK forms and three murine homologs are known and molecularly defined. In 1996, human and mouse IRAK-1 were cloned [12,17]. With hu-IRAK-2 [10] and hu-IRAK-M [18] two further human IRAKs were published in 1997 and 1999. Very recently, IRAK-4 was discovered and the human and murine form, were cloned [19] and also murine IRAK-M was identified and characterized [20].

In this paper, we describe the cloning and characterization of murine IRAK-2 (mu-IRAK-2). The complete coding sequence of mu-IRAK-2 was obtained by a database search of mouse ESTs with the human gene as template and a subsequent RACE reaction (see Materials and methods). A strong sequence homology was found in the death and kinase domains of murine and human IRAK-2. Murine IRAK-2 showed an extension of 35 amino acids at the C-terminus, the biological significance of this difference is presently unknown and remains to be determined.

As IRAK-1 knockout mice and cells derived therefrom retained residual responsiveness to IL-1 and IL-18 [21,24,33], it was suggested that the functions of hu-IRAK-M and hu-IRAK-2 were overlapping with that of hu-IRAK-1. This interpretation was supported by the observation that overexpression of hu-IRAK-2 or hu-IRAK-M in a HEK293 cell line devoid of endogenous IRAK-1,2, and -M expression allowed IL-1 dependent

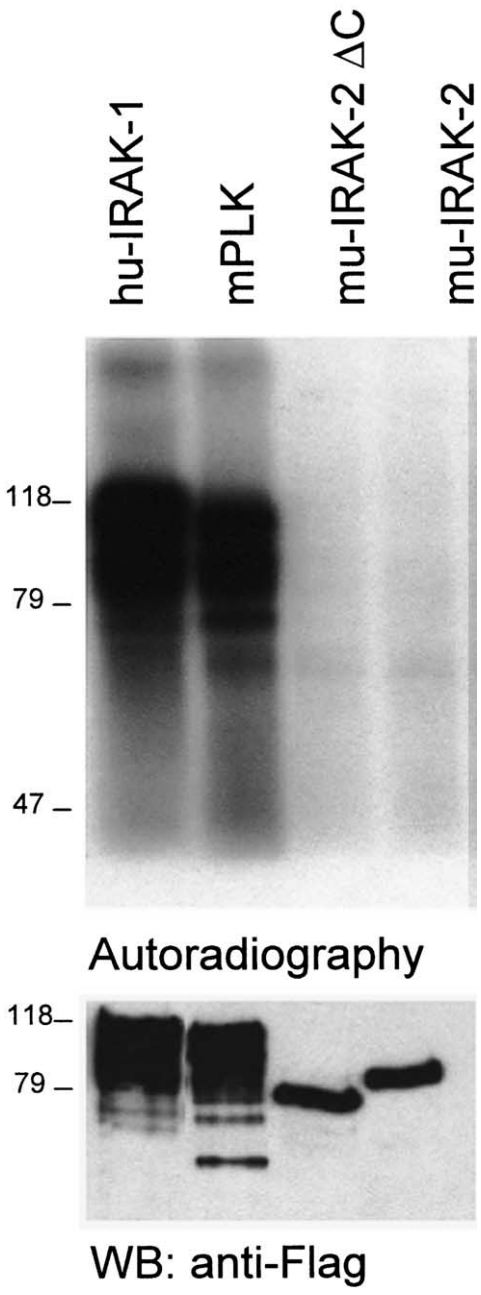

Fig. 4. Kinase activity of mu-IRAK-2. Kinase assays were performed with hu-IRAK-1 (lane 1), mPLK (lane 2), mu-IRAK-2 ΔC (lane 3), and mu-IRAK-2 (lane 4) 2×10^6 HEK293, or IRAKminus cells were transfected with 10 μg coding plasmid. Twenty-four hours after transfection cells were lysed and the proteins were precipitated with anti-Flag M2-agarose. Subsequently, the beads were used in a kinase assay with ^{32}P-labeled ATP. After SDS–PAGE the samples were analyzed autoradiographically. Both cell lines showed similar results in three independent experiments.

activation of NF-κB [18]. We compared the ability of mPLK and mu-IRAK-2 to relay the IL-1 signal in a NF-κB reporter gene assay and found a second difference between murine and human IRAK-2. Instead of stimulating IL-1 dependent NF-κB activation, as can be observed for hu-IRAK-2, overexpression of murine IRAK-2 had no effect on IL-1 dependent NF-κB activation. Whether the extended C-terminus plays a role in this phenomenon was determined with the C-truncated mu-IRAK-2 ΔC, which has a comparable C-terminus to its human counterpart. Like mu-IRAK-2, mu-IRAK-2 ΔC showed no dose-dependent stimulation of IL-1 induced NF-κB activation. These results may indicate that the human and the murine molecules may serve different purposes.

Human IRAK-1 shows strong autophosphorylation in in vitro kinase assays. In vivo the initial phosphorylation event in the activation loop of IRAK-1 kinase domain may by catalyzed by IRAK-4 as reported recently by Wesche and co-workers [19]. Subsequently, multiple phosphorylations can be observed in the regulatory ProST region identified by us recently (for a review see [25]). The consequence of these phoshorylation events are the dissociation of hyperphosphorylated hu-IRAK-1 from the upstream adaptor MyD88, allowing optimal interaction with the downstream adaptor TRAF6 and at the same time targeting it to the proteasome to become proteolytically degraded. Interestingly, the signaling function of hu-IRAK-1 was reported to be independent of its kinase activity. Thus, the kinase-inactive mutant K239S [18,26–29], was able to stimulate IL-1 dependent NF-κB activation similarly to wildtype hu-IRAK-1.

It was reported that hu-IRAK-2 showed no autophosphorylation activity in in vitro kinase assays [13]. This loss is a consequence of amino acid substitutions in the catalytic center of hu-IRAK-2, compared to hu-IRAK-1 [20]. Hu-IRAK-1 posseses an aspartic acid in position 340, which upon mutation to alanin (D340A) results in an enzymatic-inactive hu-IRAK-1 molecule [23]. At the comparable positions hu-IRAK-2 (335) and mu-IRAK-2 (333) both harbor an asparagine residue, resembling the kinase-inactive hu-IRAK-1 protein. In this study, we compared the kinase activities of mu-IRAK-2 and mu-IRAK-2 ΔC with those of human IRAK-1 and mPLK and showed that mu-IRAK-2, and mu-IRAK-2 ΔC like hu-IRAK-2, had no detectable autophosphorylation activity.

In summary, mu-IRAK-2 reveals strong homologies to its human counterpart, with respect to its tissue expression and kinase activity. However, mu-IRAK-2 also shows differences to the human molecule. Its C-terminus is longer than the hu-IRAK-2 sequence published by Muzio et al. [10] and it does not enhance IL-1 stimulated signal transduction upon overexpression. Recent data also suggest that IRAK-2 may play a different role than IRAK-1, in signal coupling of TLR4 as it preferably interacts with Mal/TIRAP [30]. The phenotype of an IRAK-2 knockout mouse will help to understand the subtle differences between IRAK-1, IRAK-2, and IRAK-M better. The identification and cloning of murine IRAK-2 is the first step in this direction.

Acknowledgments

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