

RESEARCH COMMUNICATION

Overexpression of an enzymically inactive interleukin-1-receptor-associated kinase activates nuclear factor- κ BBarbara MASCHERA^{*1}, Keith RAY^{*}, Kimberly BURNS[†] and Filippo VOLPE^{*}^{*}Glaxo Wellcome, Cell Biology Unit, Gunnels Wood Road, Stevenage SG1 2NY, U.K., and [†]Institute of Biochemistry, University of Lausanne, Ch. Des Boveresses 155, CH-10066 Epalinges, Switzerland

Upon interleukin 1 (IL-1) stimulation, the IL-1-receptor (IL-1R)-associated kinase (IRAK) is rapidly recruited to the IL-1R complex and undergoes phosphorylation. Here we demonstrate that recombinant wild-type IRAK (IRAK-WT), but not a kinase-defective mutant with Asp³⁴⁰ replaced by an asparagine residue (IRAK-Asp³⁴⁰Asn), is highly phosphorylated and is capable of auto-phosphorylation *in vitro*. Overexpression of both IRAK-

WT and IRAK-Asp³⁴⁰Asn caused activation of nuclear factor κ B, suggesting that the kinase activity of IRAK is not required outside of the IL-1R complex.

Key words: IL-1 signalling, IRAK-2, NF- κ B-inducing kinase (NIK).

INTRODUCTION

Interleukin 1 (IL-1) is a pro-inflammatory cytokine that plays a pivotal role in inflammation, immune cell regulation and induction of acute-phase proteins [1,2]. The pro-inflammatory events that follow IL-1 stimulation are often mediated by activation of specific gene products that are controlled by the transcription factor nuclear factor- κ B (NF- κ B). Recently, new components involved in the process that leads to inhibitory κ B (I κ B) phosphorylation and, consequently, nuclear translocation of NF- κ B, have been identified (for reviews, see [3–6]). However, the exact roles of individual proteins acting in this signal transduction cascade have not been defined. Following IL-1 stimulation of mammalian cells, the IL-1-receptor (IL-1R)-associated kinase (IRAK) is rapidly recruited into the IL-1R complex via its association with the IL-1R accessory protein (IL-1RAcP) [7,8], with the involvement of MyD88 [9–11]. IRAK becomes highly phosphorylated after IL-1 stimulation [12], but it is not clear whether this results from auto-phosphorylation or from phosphorylation catalysed by another kinase, possibly also associated with the IL-1R [13]. Subsequently, phosphorylated IRAK is released from the IL-1R complex and interacts with the tumour-necrosis-factor (TNF)-receptor-associated factor-6 (TRAF6) [14]. TRAF6 also interacts with the NF- κ B-inducing kinase (NIK), which participates in the regulation of the I κ B kinase complex, implicated in NF- κ B activation [15]. The importance of IRAK kinase activity for signal transduction remains unclear, since no direct evidence for phosphorylation of potential substrates, such as TRAF6 and/or NIK, has been reported.

Recent studies on fibroblast cells from IRAK gene knock-out mice have confirmed that IRAK plays an important role in IL-1 signalling [16]; nevertheless, the importance of IRAK catalytic activity for activation of NF- κ B is still not clear.

IRAK represents an attractive target for the development of

specific inhibitors to block the pro-inflammatory actions of IL-1. To investigate the role of the kinase activity of IRAK in activation of the NF- κ B signalling pathway, we have assessed the effects of a kinase domain mutation on the ability of IRAK to undergo auto-phosphorylation, and to function as an activator of NF- κ B-dependent gene expression.

EXPERIMENTAL**Cell Cultures**

Mammalian cell cultures were carried out at 37 °C under 5% CO₂. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-calf serum and penicillin/streptomycin (100 μ g/ml of each). The Sf9 insect cells were grown at 28 °C as suspension cultures in TC100 medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% (v/v) fetal-calf serum and penicillin/streptomycin (100 μ g/ml of each).

Constructs

The preparation of full-length IRAK cDNA has been described previously [7]. The cDNA species featuring the Asp³⁴⁰ → Asn substitution (IRAK-Asp³⁴⁰Asn cDNA) was created from wild-type IRAK (IRAK-WT) cDNA by QuickChange[™] site-directed mutagenesis (Stratagene, Cambridge, U.K.), using the following oligonucleotides (where altered nucleotides are underlined): sense, 5'-GCCTCATCCATGGAACATCAAGAGTTCCAA-CG-3'; antisense, 5'-CGTTGGAACTCTTGATGTTTCCATG-GATGAGGC-3'. Constructs of NIK-WT and a double mutant of NIK (NIK-Lys⁴²⁹Ala/Lys⁴³⁰Ala) were kindly provided by Dr. A. Kaptein (Glaxo Wellcome).

Abbreviations used: IL-1, interleukin 1; NF- κ B, nuclear factor- κ B; I κ B, inhibitory κ B; IL-1R, IL-1 receptor; IRAK, IL-1-receptor-associated kinase; IL-1RAcP, IL-1R accessory protein; TNF, tumour necrosis factor; TRAF6, TNF-receptor-associated factor-6; NIK, NF- κ B-inducing kinase; IRAK-WT, wild-type IRAK; IRAK-Asp³⁴⁰Asn, a mutant of IRAK featuring an Asp³⁴⁰ → Asn substitution; DTT, dithiothreitol; TK, thymidine kinase; sAP, secreted alkaline phosphatase.

¹ To whom correspondence should be addressed (e-mail bm45459@GlaxoWellcome.co.uk).

Cloning and expression of IRAK in insect cells

The IRAK-WT and IRAK-Asp³⁴⁰Asn cDNAs were subcloned into the *Eco*RI and *Xba*I sites of the pfastBac HTa vector and transposed site-directly into baculovirus shuttle vector (bacmids) using the Bac-to-BacSM Baculovirus expression system (Gibco BRL). Sf9 insect cells were transfected with either the recombinant bacmids or a non-recombinant virus as a negative control. After 48 h of infection, at a multiplicity of infection (m.o.i.) of 3, the cells were harvested and lysed in a buffer containing 50 mM Hepes, pH 7.9, 250 mM NaCl, 5 mM dithiothreitol (DTT), 1 mM EDTA, 20 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μ g/ml leupeptin, 0.1% (v/v) Nonidet P40 and 10% (v/v) glycerol. Soluble fractions were used for Western blot analysis with a polyclonal antibody raised against IRAK (Calbiochem, Bad Soden, Germany) at a dilution of 1:5000, and for *in vitro* kinase assays.

Phosphatase treatment of cell extracts

Soluble fractions from HEK-293 cells transfected with empty vector (pcDNA3) or pcDNA3 containing either IRAK-WT or IRAK-Asp³⁴⁰Asn were obtained in lysis buffer consisting of 50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 2 mM MgCl₂ and protease inhibitors (Boehringer Mannheim, Mannheim, Germany). Protein concentration was measured using the Bradford method (Bio-Rad, Hemel Hempstead, Herts., U.K.). Protein (15 μ g per sample) was diluted 2-fold with assay buffer containing 50 mM Tris/HCl, pH 7.6, 0.1 mM CaCl₂ and 1 mM MgCl₂. The samples were incubated at 37 °C in the presence of 40 μ -units of alkaline phosphatase PP-2A₁ (Upstate Biotechnology, Lake Placid, NY, U.S.A.) for 15 min, 60 min, 180 min and 20 h. Reactions were stopped by addition of 2 \times SDS sample buffer, followed by heating for 5 min and separation with SDS/PAGE, using 4–20% polyacrylamide Tris/glycine gels (Novex, San Diego, CA, U.S.A.), and immunoblot analysis was then performed with anti-IRAK antibody (Calbiochem).

In vitro kinase assays

Kinase assays were performed using either 10 μ l of soluble fractions from Sf9 cells prepared as described above, with equal amounts of protein (20 μ g), or 10 μ l of purified histidine-tagged IRAK (or HIS-IRAK). Addition of kinase buffer [20 mM Tris/HCl (pH 7.6)/1 mM DTT/20 mM MgCl₂/20 mM β -glycerophosphate/1 mM sodium orthovanadate/1 mM benzamidin/0.4 mM PMSF/5 μ M unlabelled ATP/5 μ Ci of [γ -³²P]ATP (5000 Ci/mmol)], in a final volume of 20 μ l, was followed by incubation of the samples at 30 °C for 45 min. Individual reactions were terminated by the addition of SDS sample buffer and boiling. The proteins were separated on 4–20% gels, and autoradiograms were prepared from dried gels exposed to HyperfilmSM ECLSM (Amersham Life Science, Little Chalfont, Bucks., U.K.) film at –70 °C for a period of 20 min to 1 day.

Reporter assays

For the NF- κ B activation assays by IRAKs, 3 \times 10⁶ NIH-3T3 cells were seeded into 25-cm² flasks. Cells were transfected the following day using the calcium phosphate precipitation method (Stratagene) with 2 μ g of a plasmid (pTK-sAP) comprising the minimal thymidine kinase (TK) promoter, containing four tandem NF- κ B sites upstream of the secreted alkaline phosphatase gene [(NF- κ B)₄-TK-sAP] [17], 3 μ g of Rous-sarcoma-virus- β -lactamase (RSV- β lac) plasmid and the indicated amounts of

expression constructs (see Figure 2). The total DNA concentration was kept constant by supplementation with empty vector.

At 24 h post-transfection, the cells were divided into 6-well plates and were stimulated the following day, where indicated, with IL-1 β (10 pM) for 16 h. The medium from the cells was used for secreted alkaline phosphatase (sAP) and β -lactamase assays; the cells were harvested for Western blot analysis.

The sAP assay was performed as described previously [17]. The β -lactamase activity was determined in a mixture containing 50 μ l of culture medium and 150 μ l of buffer containing 200 μ g/ml nitrocefine, 25 mM KH₂PO₄/NaKHPO₄, pH 7.0 and 0.05% (v/v) Triton X-100. Samples were incubated at 37 °C until a brown colour developed, and then A₄₉₀ was measured. These values were used to normalize the efficiency of transfection.

For the NF- κ B reporter assays used to detect inhibition of NF- κ B activation by NIK dominant negative, 1 \times 10⁶ HEK-293T cells were seeded into 35-mm dishes. Cells were transfected by the calcium phosphate method (Stratagene) with 0.5 μ g of TK-sAP plasmid, 0.5 μ g of RSV- β gal and 50 ng of the indicated expression constructs, in the presence or absence of 2 μ g of NIKdn (NIK-Lys⁴²⁹Ala/Lys⁴³⁰Ala) plasmid (see Figure 3). The total DNA concentration was kept constant by supplementation with empty vector. At 24–48 h post-transfection, the medium from the cells was used for the sAP assay, while the cells were harvested, lysed and subjected to β -galactosidase assay as described by Burns et al. [11].

RESULTS AND DISCUSSION

Wild-type IRAK, but not the kinase-defective mutant IRAK-Asp³⁴⁰Asn, undergoes auto-phosphorylation

In order to create a kinase-defective mutant of IRAK, we mutated the aspartate residue, which is highly conserved for catalytic function within kinase subdomain VIb, to an asparagine (Asp³⁴⁰Asn).

IRAK-WT, expressed in HEK-293 and NIH-3T3 cells, was detected on Western blots as a broad band, ranging in molecular size from \approx 80 to 100 kDa (Figure 1A; also see Figure 2C). In contrast, the migration of mutant IRAK-Asp³⁴⁰Asn was close to that expected for the native protein with a predicted molecular weight of 76 kDa. A faint upper band was also recognized using the anti-IRAK antibody (Figures 1A and 2C).

Confirmation that the mobility shift observed for IRAK-WT was due to hyperphosphorylation was obtained by treating the cell extracts with protein phosphatase 2A₁ (Figure 1A). This treatment changed the mobility of the IRAK-WT band such that it migrated to a position close to the expected molecular size, although the migration of IRAK-Asp³⁴⁰Asn was not affected. These results suggest that the catalytic activity of IRAK is necessary for generation of the slower-migrating forms of IRAK-WT, and are consistent with previous studies in which IRAK undergoes a marked mobility shift on SDS/PAGE, following activation by IL-1 [12].

To assess directly the kinase activities of the native and mutant forms of IRAK, *in vitro* kinase assays were performed using extracts from insect cells expressing the different proteins. Following incubation with [γ -³²P]ATP, cell extracts were subjected to SDS/PAGE and autoradiography. A broad band corresponding to 90–120 kDa was detected specifically in samples derived from cells infected with the recombinant virus expressing histidine-tagged IRAK-WT; this band co-migrated with IRAK immunoreactivity on a Western blot (Figure 1B, lane 2). The fusion of the polyhistidine tag to IRAK accounts for a slower migration, as determined by SDS/PAGE, compared with un-

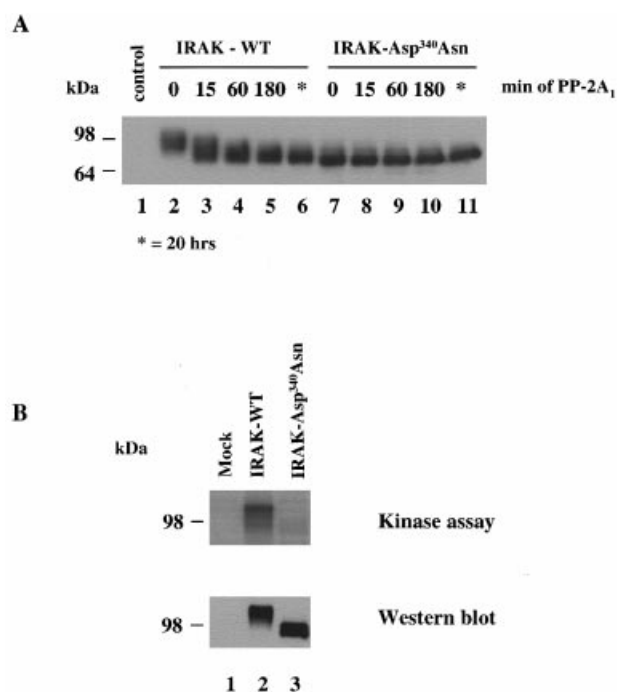


Figure 1 Effects of a single point mutation (Asp³⁴⁰Asn) on the phosphorylation state and activity of IRAK

(A) Dephosphorylation assay: extracts from transfected HEK-293 cells expressing IRAK-WT (lanes 2–6) or IRAK-Asp³⁴⁰Asn (lanes 7–11) were incubated in the presence of protein phosphatase 2A₁ (PP-2A₁) for the times indicated (lanes 3–6 and 8–11 for IRAK-WT and IRAK-Asp³⁴⁰Asn respectively), or they were left untreated (lanes 2 and 7). The control lane represents untransfected HEK-293 cell extracts. Equal amounts of protein were separated by using SDS/PAGE, and were immunoblotted with anti-IRAK antibody. (B) Protein extracts derived from insect cells infected with non-recombinant virus (lane 1), with recombinant IRAK-WT virus (lane 2) and with recombinant IRAK-Asp³⁴⁰Asn virus (lane 3) were subjected to *in vitro* kinase assay and Western blot analysis using anti-IRAK antibodies.

tagged IRAK (Figures 1A and 2C). No equivalent ³²P-labelled band was detected in assays using extracts from IRAK-Asp³⁴⁰Asn-infected cells, although Western blots confirmed the expression of the protein (Figure 1B, lane 3). These results indicate that the mutation Asp³⁴⁰Asn resulted in a loss of IRAK auto-phosphorylation activity.

In a kinase assay with the affinity-purified histidine-tagged IRAK-WT, an equivalent ³²P-labelled band of about 120 kDa was detected (results not shown), suggesting that IRAK undergoes auto-phosphorylation, rather than being phosphorylated by another kinase present in the insect cell extracts.

Both IRAK-WT and IRAK-Asp³⁴⁰Asn activate NF- κ B-dependent transcription via NIK

In order to assess the importance of the kinase activity of IRAK for IL-1 signal transduction, we investigated the effects of overexpressing either the IRAK-WT or IRAK-Asp³⁴⁰Asn on cytokine-inducible, NF- κ B-dependent reporter gene expression (Figure 2). Mouse 3T3 cells were co-transfected with plasmids for expression of IRAK, together with reporter genes to monitor NF- κ B activation and transfection efficiency. Subsequently, basal and IL-1-inducible expression of the NF- κ B-dependent alkaline phosphatase was assessed. Mock-transfected 3T3 cells responded to IL-1 β (10 pM) stimulation, giving a 4–5-fold increase in sAP production. Effects of the IRAK-WT on NF- κ B activation

varied, depending on the level of expression achieved; IRAK expression alone increased reporter gene activation from 3- to 16-fold. At the highest dose of 5 μ g of IRAK-WT, NF- κ B activation was lower than that observed with either 0.5 μ g or 0.05 μ g. Because our data are normalized using an internal control for transfection efficiency, we would expect that any possible toxic effect due to overexpression of IRAK should have been eliminated; we are currently investigating the reason for this observation.

At lower expression levels (Figure 2A), IRAK activated NF- κ B reporter gene production more strongly, indicating an absence of a direct correlation between expression of phosphorylated IRAK and activation of the pathway. The maximal effect of IRAK overexpression on the production of the NF- κ B reporter gene was greater than that elicited by IL-1 alone, and was not further increased in the presence of IL-1. These results suggest that IRAK overexpression alone can fully activate a pathway that leads to NF- κ B activation.

We then investigated the effects of IRAK-Asp³⁴⁰Asn expression on IL-1 signalling in 3T3 cells. Unexpectedly, expression of this form of IRAK strongly induced basal NF- κ B-dependent reporter gene expression (Figure 2B); the levels of activation were greater than those obtained with IRAK-WT, although equivalent amounts of protein were detected on Western blots.

As observed for IRAK-WT, at the highest dose of IRAK-Asp³⁴⁰Asn used, NF- κ B reporter gene induction was less than that observed with 0.5 μ g and 0.05 μ g. Further stimulation of the reporter gene was not evident in the presence of IL-1, at the concentrations used for IRAK-Asp³⁴⁰Asn expression. As shown in Figure 2(C), lanes 5–7, the form of IRAK-Asp³⁴⁰Asn expressed in 3T3 cells corresponded to the native 76 kDa form, with a slower-migrating form detectable at the highest doses. Hyperphosphorylation of IRAK-Asp³⁴⁰Asn was not detectable, suggesting that this modification is due to an intramolecular event requiring intrinsic kinase activity, rather than intermolecular phosphorylation by endogenous IRAK or another kinase.

These results suggest that the auto-phosphorylation of IRAK is not required for activation of signal transduction events downstream of the IL-1R complex. Given the capacity of IRAK to interact with itself [7], we cannot exclude the possibility that ectopic expression of IRAK-Asp³⁴⁰Asn might have caused 'activation' via its association with endogenous IRAK. We are currently investigating these possibilities.

IRAK-WT and IRAK-Asp³⁴⁰Asn overexpression might induce kinase-independent activation of the pathway via aggregation of other signalling molecules, or sequestration of inhibitory proteins regulating downstream signalling events.

It has been shown previously that hyperphosphorylation of endogenous IRAK leads to proteasome targeting [19]. Here we demonstrate that hyperphosphorylation of ectopically expressed IRAK-WT is not related to NF- κ B activation. Taken together, these observations suggest that auto-phosphorylation activity of IRAK might be involved in the regulation of signal termination, rather than signal transduction.

In order to confirm that both IRAK-WT and kinase-defective IRAK-Asp³⁴⁰Asn induce NF- κ B activation via the IL-1 signalling cascade, we assessed whether a dominant-negative mutant of NIK could block that activation. 293T cells were co-transfected with IRAK-WT, IRAK-Asp³⁴⁰Asn and NIK-WT plasmids in the presence or absence of a construct expressing a mutated form of NIK (NIK-Lys⁴²⁹Ala/Lys⁴³⁰Ala), previously shown to block IL-1-induced NF- κ B [15]. Dominant-negative NIK inhibited approx. 60% of the NF- κ B activation induced by both IRAK-WT and IRAK-Asp³⁴⁰Asn, whereas it blocked almost completely the sAP reporter gene expression induced by NIK-WT (Figure

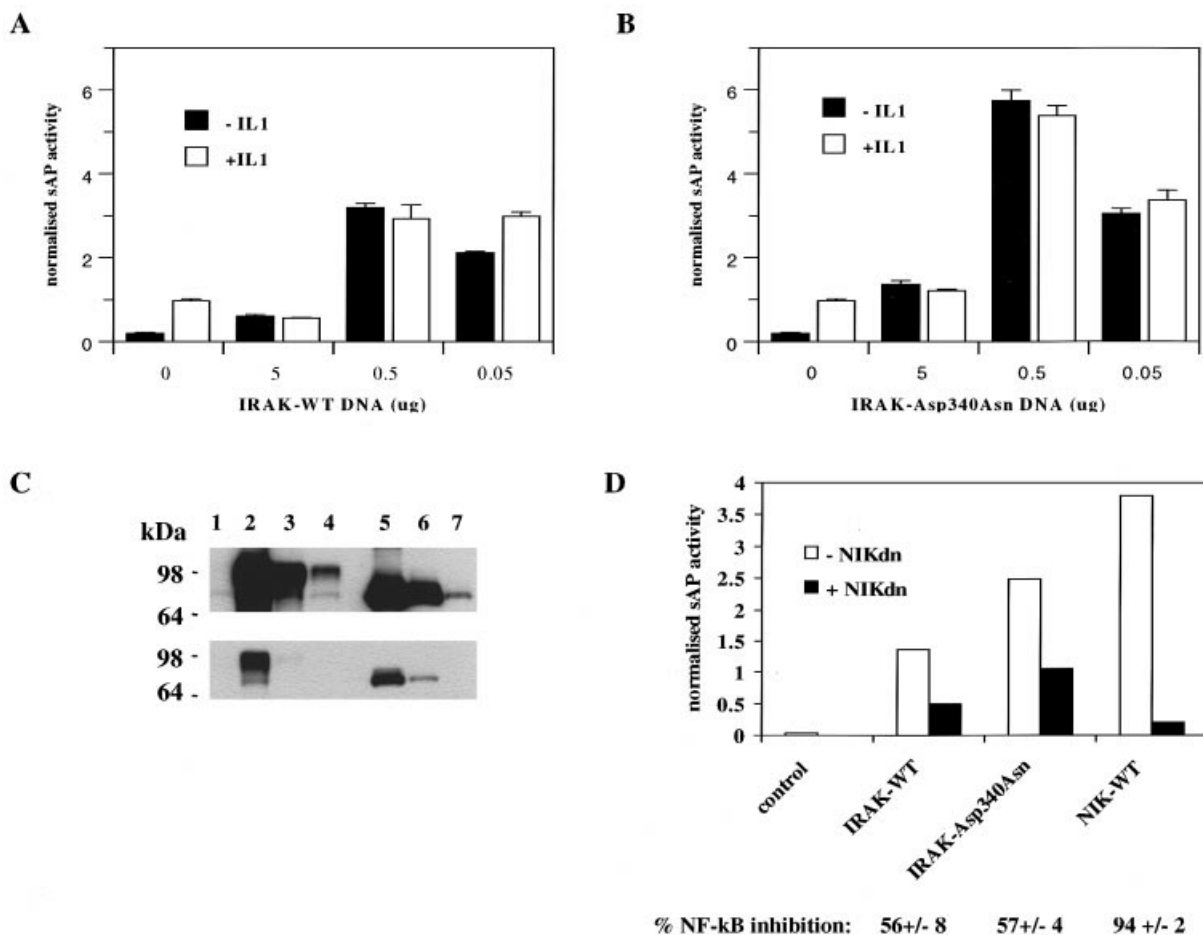


Figure 2 Effects of IRAK-WT and IRAK-Asp³⁴⁰Asn overexpression on NF-κB-driven reporter gene expression

(A) Activation of NF-κB reporter gene by IRAK-WT in the absence and presence of IL-1 (10 pM). NIH-3T3 cells were transiently co-transfected with a (NF-κB)₄-TK-sAP reporter plasmid, β-lactamase and the indicated amounts of IRAK-WT expression vector. Data represent sAP activity, normalized for β-lactamase expression, and are shown for a representative experiment. Each experiment was performed in quadruplicate, and the error bars indicate the S.E.M. for a representative experiment. (B) Activation of NF-κB reporter gene by IRAK-Asp³⁴⁰Asn in the absence or presence of IL-1. This was measured under the same experimental conditions as those described in (A). (C) Western blotting of cell extracts derived from one of the NF-κB reporter gene experiments described above, using antibodies raised against IRAK. Two different exposures of the film are shown. (D) Dominant-negative NIK blocks NF-κB activation induced by both IRAK-WT and IRAK-Asp³⁴⁰Asn. HEK-293T cells were transiently co-transfected with (NF-κB)₄-TK-sAP reporter plasmid, β-galactosidase and 50 ng of each of the indicated expression vectors, in the absence or presence of 2 μg of NIKdn (NIK-Lys⁴²⁹Ala/Lys⁴³⁰Ala). Data represent sAP activity, normalized for β-galactosidase, and are shown for a representative experiment. The values of the NF-κB inhibition are expressed as percentages ± S.D., and they are derived from three different experiments.

																10					20									
IRAK-2	G	E	L	I	H	S	N	V	K	S	S	N	V	L	L	D	Q	N	L	T	P	K	L	A	H	.	P	M	A	H
IRAK	S	P	S	I	H	G	D	I	K	S	S	N	V	L	L	D	E	R	L	T	P	K	L	G	D	F	G	L	A	R
Pelle	G	T	P	I	H	G	D	I	K	P	A	N	I	L	L	D	Q	C	L	Q	P	K	I	G	D	F	G	L	V	R
Pti-1	Q	P	H	I	H	R	D	I	K	S	S	N	I	L	L	F	D	D	D	V	A	K	I	A	D	F	D	L	S	N
Pto	T	R	A	I	H	R	D	V	K	C	T	N	I	L	L	D	E	N	F	V	P	K	I	T	D	F	G	I	S	K

Figure 3 IRAK-Asp³⁴⁰Asn contains the same amino acid substitution as that found in IRAK-2

Multiple-amino-acid sequence alignment of the kinase subdomains VIIb and VIIc of IRAK-2, IRAK, Pelle, Pti-1 and Pto. The alignment was performed using Clustal software, and modified manually. The single-letter amino acid code has been used. Amino acids that are conserved among all the five proteins are represented in boxes. The two aspartate residues highly conserved in protein kinases [26] are shown in bold; these are substituted in IRAK-2, as shown by white lettering in black boxes.

2D). These results confirm that the kinase-defective IRAK-Asp³⁴⁰Asn, as well as IRAK-WT, is still capable of activation of the IL-1 signalling cascade. Moreover, they suggest that activation of NF-κB by both forms of IRAK is also mediated via NIK-independent signalling pathways.

IRAK-2 is a putative inactive kinase

Recently, Muzio et al. [9] have discovered a novel molecule, designated IRAK-2, on the basis of its sequence and functional similarities to IRAK. Both IRAK and IRAK-2 are recruited to

the IL-1R complex, and both appear to act upstream of TRAF6 on the pathway regulating NF- κ B activation. Although IRAK-2 is predicted to contain a kinase-like domain, evidence that two of the highly conserved aspartates in the 'active site' are substituted (Figure 3) suggests that the protein does not possess this enzymic activity. The invariant aspartate residue within the catalytic loop (subdomain VIb), which most probably serves as the catalytic base for the reaction, and the aspartate residue within the DFG loop (subdomain VII) are missing in IRAK-2.

The catalytic aspartate residue is highly conserved in all of the 390 protein kinases listed in the Protein Kinase Resource database [20], with the exception of two, i.e. erbB3, which possesses impaired kinase activity [21,22], and TMKL1, which is likely to be inactive [23].

Interestingly, the residue found to occur natively in IRAK-2 that corresponds to the first of the two conserved catalytic aspartyl residues in IRAK is an asparagine. These observations, together with the fact that the equivalent mutation (Asp \rightarrow Asn) results in a kinase-defective form of IRAK (IRAK-Asp³⁴⁰Asn), strongly suggest that IRAK-2 does not possess kinase activity. Since IRAK-2, like IRAK, has been implicated in IL-1R signal transduction, it would appear that the native form of IRAK-2 could perform its role without the requirement for kinase activity. These observations, together with the experiments reported above for IRAK, bring into question the requirement of the kinase enzymic activities of IRAK, at least outside of the IL-1R complex, for NF- κ B activation.

Properties of IRAK and IRAK-2 resemble those of the serine/threonine kinases involved in the TNF-signalling pathway, i.e. RIP and the recently identified RIP-2, the catalytic activities of which are not required for NF- κ B activation [24,25].

In view of the current interest in the development of IRAK inhibitors as drugs to treat inflammatory diseases, we suggest that further studies should focus on the molecular mechanism(s) of IRAK phosphorylation.

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