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

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The interleukin-1 (IL-1)-receptor-associated kinase (IRAK-1) is essential for IL-1-stimulated nuclear factor κ B (NF- κ B) activation. To study the role of IRAK-1 in IL-1 β signalling, we have generated a set of IRAK-1 variants that express distinct domains of IRAK-1 either alone or in combination and have examined their effects on an NF- κ B-responsive reporter in HeLa cells. Unlike full-length IRAK-1, the deletion mutants were unable to activate NF- κ B in the absence of cytokine stimulation. However, an IRAK-1 variant lacking only the N-terminal domain retained the ability of the full-length protein to potentiate both IL-1 β and tumour necrosis factor α (TNF α)-induced NF- κ B activation. In contrast, expression of the N-terminus or the C-terminus of IRAK-1, or a fusion protein incorporating both domains, inhibited both IL-1 β - and TNF α -induced effects. Expression of an IRAK-1 variant lacking only the C-terminal domain preferentially inhibited IL-1 β versus TNF α -induced

NF- κ B activation. These data suggest that the C-terminal domain may link IRAK-1 to downstream signalling components common to both the IL-1 and TNF pathways. Furthermore, we have demonstrated that endogenous IRAK-1 becomes phosphorylated upon IL-1 β treatment and can be detected along with NF- κ B essential modulator (NEMO) and I κ B kinase β (IKK β) in high-molecular-mass complexes of 600–800 kDa. Moreover, IRAK-1 could be detected in NEMO immunoprecipitates from IL-1 β -stimulated cells. We conclude that IRAK-1 mediates IL-1 β signal transduction through a ligand-dependent association of IRAK-1 with the IKK complex.

Key words: I κ B kinase (IKK), interleukin-1 signal transduction, tumour necrosis factor (TNF), ubiquitination.

INTRODUCTION

The proinflammatory effects of cytokines such as interleukin-1 β (IL-1 β) and tumour necrosis factor- α (TNF α) are mediated at least in part through activation of nuclear factor κ B (NF- κ B), a transcription factor which commonly exists as a heterodimer of 50 and 65 kDa subunits [1]. In most cells, NF- κ B is sequestered in the cytoplasm through association with a family of inhibitor proteins, the I κ Bs, which mask the nuclear localization signal (NLS) present within the p65 subunit of NF- κ B [2,3]. Following cytokine stimulation, I κ B α is phosphorylated rapidly on Ser³² and Ser³⁶, leading to its ubiquitination and targeting for degradation by the 26 S proteasome [4–8]. This unmasks the NLS of NF- κ B, allowing it to translocate into the nucleus and initiate the transcription of many genes involved in inflammatory and immune responses [9,10].

The cytokine-induced phosphorylation of I κ B molecules is mediated by an 800 kDa I κ B kinase (IKK) complex. The regulation and exact composition of the IKK complex remains unclear, but it is believed to contain the protein kinases IKK α and IKK β [11,12] and a regulatory subunit called IKK γ or

NF- κ B essential modulator (NEMO) [13–15]. Knock-out studies have revealed that IKK β (rather than IKK α) is essential for NF- κ B activation in response to proinflammatory stimuli [16]. In the absence of NEMO, proinflammatory cytokines such as IL-1 fail to activate the IKK complex or NF- κ B [14]. It may be that NEMO connects the IKK complex to upstream activators; however, currently these signalling pathways are poorly defined.

The binding of IL-1 to the extracellular domain of the IL-1RI initiates the assembly of a receptor signalling complex through the engagement of the IL-1 receptor accessory protein (IL-1RAcP) [17–19]. The serine/threonine kinase IRAK-1 is recruited into the activated IL-1RI complex by a mechanism involving the adapter molecule MyD88 [20,21]. Recently it has been reported that an additional component termed ‘Tollip’ may also be involved in initiating this process [22].

Receptor-associated IRAK-1 becomes highly phosphorylated [20,21,23], but the regulatory significance and mechanisms responsible for this process remain to be fully established. It is thought that IRAK-1 phosphorylation may influence its subsequent dissociation from the receptor complex and movements into the cytoplasm where it binds to TNF-receptor-associated

Abbreviations used: IL-1, interleukin-1; TNF, tumour necrosis factor; IL-1RI, type 1 interleukin 1 receptor; TNFR1, type 1 TNF receptor; IL-1RAcP, interleukin-1 receptor accessory protein; IRAK, interleukin-1-receptor-associated kinase; NEMO, Nuclear factor κ B (NF- κ B) essential modulator; IKK, I κ B kinase; RIP, receptor-interacting protein; sAP, secreted alkaline phosphatase; NLS, nuclear localization signal; SODD, silencer of death domains; TRADD, TNF-receptor-associated death domain protein; TK, thymidine kinase; RSV, Rous sarcoma virus; β lac, β -lactamase; TAK-1, TGF β activated kinase 1.

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factor 6 (TRAF6) [24]. Subsequent events leading to IKK activation remain to be determined.

The type 1 TNF receptor (TNFR1; p55) is responsible for TNF signalling in most cell types [25]. The intracellular domain of the p55 TNFR1 contains a 'death domain', which allows it to interact with other death-domain-containing proteins such as SODD (silencer of death domains) in the absence of cytokine stimulation. Binding of TNF induces TNFR1 dimerization or trimerization, leading to the rapid dissociation of SODD from the receptor complex, allowing the TNF-receptor-associated death domain protein (TRADD), to bind to the receptor through the same region [26,27]. The TNFR1-TRADD complex is able to induce NF- κ B activation through interaction with the adapter molecule RIP (receptor-interacting protein) [28], which binds to NEMO upon TNF stimulation and recruits the IKK-signalsome to the p55 TNF receptor [29] [the IKK-signalsome, a term coined by Michael Karin and others, is a high-molecular-mass (800 kDa) cytoplasmic protein complex with IKK activity; the exact composition of this IKK complex remains unclear, but it is known to contain catalytic subunits, IKK α and IKK β [11,12] and a regulatory subunit called IKK γ [13–15]].

To understand more about how IRAK-1 functions in IL-1 β signalling, we have generated a set of constructs expressing distinct domains of IRAK-1 either alone or in combination. We have examined the effects of overexpression of these constructs on NF- κ B activation in HeLa cells using reporter gene assays. In addition, we have used gel-filtration analysis and immunoprecipitation experiments to study the composition of IRAK-1-containing complexes in early signal transduction.

EXPERIMENTAL

Biological reagents and cell culture

Recombinant human IL-1 β (1.2×10^7 i.u./mg) was expressed in *Escherichia coli* and purified as described previously [30]. Recombinant human TNF α was purchased from R & D systems (Abingdon, Oxfordshire, U.K.). The anti-FLAG monoclonal antibody M2 was purchased from Sigma (FLAG is the epitope Asp-Tyr-Lys-Asp-Asp-Asp-Lys). The rabbit anti-NEMO and goat anti-IKK β polyclonal antibodies were purchased from Santa Cruz (via their UK supplier Autogen Bioclear, Calne, Wilts., U.K.). Polyclonal anti-rabbit IgG, anti-goat IgG and anti-mouse IgG-conjugated horseradish peroxidase antibodies were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). The rabbit anti-IRAK polyclonal antibody was raised in-house against the peptide Glu-Gln-Asp-Arg-Gln-Gly-Pro-Glu-Glu-Ser-Asp-Glu-Phe-Gln-Ser (IRAK-1 residues 698–712). HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin/gentamicin (100 μ g/ml of each). Mammalian cell cultures were carried out at 37 °C under 5% CO₂.

Cloning and expression vectors

Full-length IRAK-1 cDNA, IRAK-1-FL(1–712) was obtained by reverse-transcription PCR from mRNA isolated from a HEK293 cell line [31]. All constructs used for the transient transfection studies described were cloned into *Eco*R1/*Xba*I-digested plasmid pBKRSV (Stratagene). IRAK-1-FL(1–712) was digested out of the pcDNA3.1 vector and ligated into the pBKRSV vector (the number ranges refer to sections of the IRAK sequence, and the initials before the sequences indicate constructs encoding the following regions of IRAK-1: FL, full-length; CT, C-terminal region; K, kinase domain; NT, N-terminal region; NK, N-terminal and kinase domains). IRAK-1-

CT(533–712), IRAK-1-K(212–532) and IRAK-1-KC(212–712) were constructed by inserting PCR-generated cDNA fragments into pBKRSV. Using the full-length wild-type IRAK-1/pBKRSV plasmid as template, IRAK-1-NT(1–211) and IRAK-1-NK(1–532) were generated by introducing stop codons/*Kpn*I sites (TGA) immediately after the relevant domains. The 3'-untranslated region on IRAK-1-NT(1–211) was removed by *Kpn*I restriction digestion, followed by re-ligation. In order to clone the IRAK-1-NC(1–211:533–712) construct, which lacked the entire kinase domain, *Eco*RV sites were incorporated at each end of the kinase domain by site-directed mutagenesis. Restricted digestion allowed the kinase domain to be removed and the remaining plasmid was re-ligated to generate the fusion protein. The IRAK-1-NT(1–211), IRAK-1-NK(1–532) and IRAK-1-K(212–532) constructs were epitope-tagged with FLAG using QuikChange Site-Directed Mutagenesis according to the manufacturer's (Stratagene) protocol.

Reporter assays

HeLa cells (4.5×10^5) were seeded into 12-well plates. The following day, cells were transfected by calcium phosphate precipitation (Stratagene). A 0.3 μ g portion 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 (sAP) gene [(NF- κ B)₄-TK-sAP], 0.2 μ g of Rous-sarcoma-virus- β -lactamase (RSV- β lac) plasmid and the indicated amounts of expression constructs (see the Figure legends) were transfected into the cells. The total DNA concentration was kept constant at 1.6 μ g/well by adding empty vector. After 24 h, cells were either left untreated or stimulated with TNF α (15 ng/ml) or IL-1 β (10 pM). At 30 h following treatment, the media from the cells were collected and reporter gene activities determined (sAP and β lac assays). Additionally, cells were harvested directly into 2 $\times$ SDS sample buffer for Western-blot analysis. Readings for induced sAP activity were normalized for transfection efficiency based on production of the co-transfected constitutively active RSV- β lac reporter gene. For the sAP assay, media were heat-treated at 60 °C for 30 min to inactivate the endogenous AP. A 25 μ l portion of the culture medium from each sample was then combined with 175 μ l of DEA buffer (1 M diethanolamine/HCl, 0.28 M NaCl, 0.5 mM MgCl₂ and 5 mM *p*-nitrophenyl phosphate, pH 9.85, at 22 °C). Samples were incubated at 37 °C for 20–40 min and the absorbance read at 405 and 650 nm. To determine β lac activity, 50 μ l of each sample was incubated at 37 °C with 150 μ l of buffer containing 200 μ g/ml nitrocefin, 25 mM KH₂PO₄/NaKHPO₄, pH 7.0, and 0.05% (v/v) Triton X-100. Once a yellow coloration had developed, the absorbance was determined at 490 nm. Reaction conditions were established to ensure that, for all samples, comparative readings were taken where kinetics of colour development were linear.

Phosphatase treatment of cell extracts

Cells were lysed on ice in 100 μ l of buffer containing 50 mM Tris/HCl (pH 7.6), 1 mM EDTA, 150 mM NaCl, 1 mM MgCl₂, 0.1 mM MnCl₂, 0.5 mM PMSF, 60 mM mercaptoethanol, protease inhibitors (Boehringer-Mannheim, Mannheim, Germany) and 0.1% Nonidet P40. Aliquots of lysates (30 μ l each) were incubated at 37 °C with or without 40 μ units of protein phosphatase PP-2A₁ (Upstate Biotechnology, Lake Placid, NY, U.S.A.) for 60, 120 and 180 min. Reactions were terminated by addition of 2 $\times$ SDS sample buffer, followed by heating of the samples at 85 °C for 5 min. Proteins were separated using SDS/PAGE on 4–20% polyacrylamide/Tris/glycine gels

Figure 1 IRAK-1 variants

The structure of each of the IRAK-1 constructs used is illustrated. The 'hairpin' indicates an internally deleted region. The positions of the death domain (bars stippled with dots, left), the central kinase domain (black bars, central), and the C-terminal domain (bars stippled with vertical lines, right) of IRAK-1 are shown. Numbering refers to the amino acid position of the full-length IRAK-1 protein.

(Novex, San Diego, CA, U.S.A.) and IRAK detected using immunoblot analysis.

Immunoblotting

For Western-blot analysis of whole-cell lysates, proteins were separated by SDS/PAGE, electroblotted on to nitrocellulose

membranes (Bio-Rad), and incubated with the indicated primary and secondary antibodies (see the Figure legends) to detect specific immunoreactive bands. The membranes were then developed using the enhanced-chemiluminescence system (ECL[®]; Amersham).

Co-immunoprecipitation

HeLa cell cytoplasmic extracts were subjected to immunoprecipitation with anti-NEMO or anti-M2 FLAG antibodies. Lysates were precleared using 40 μ l of GammaBind G-Sepharose beads (66% slurry; Amersham Pharmacia). A 2–5 μ l portion of each antibody was added to the lysates for 2–3 h. Lysates were subsequently incubated overnight at 4 °C with 40 μ l of a 66% slurry of GammaBind G-Sepharose beads. The beads were then washed five times with lysis buffer and the precipitates separated on Tris/glycine gels and analysed by Western blotting.

Size-exclusion chromatography

HeLa cells (5×10^7) were lysed in 500 μ l of buffer A [50 mM Hepes, pH 7.9, 500 mM NaCl, 5 mM dithiothreitol (DTT), 1 mM EDTA, 20 mM glycerophosphate, 10 mM NaF, 10 mM *p*-nitrophenyl phosphate, 0.5 mM sodium orthovanadate, 0.5 mM PMSF, 0.25% (v/v) Nonidet P40] for 1 h at 4 °C. After centrifugation for 30 min at 10000 *g*, the supernatant was recovered and gel-filtration chromatography was carried out on a Superdex 200HR 10/30 column (Pharmacia). The column was precalibrated with thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B-12 (1350 Da). Fractions (500 μ l) of the cell extract were collected and aliquots analysed directly by immunoprecipitation or Western blotting.

Figure 2 Effects of full-length wild-type IRAK-1 and the IRAK-1 variants on NF- κ B reporter gene activity in the absence of cytokine stimulation

HeLa cells (12-well plates) were co-transfected with 0.3 μ g/well of an (NF- κ B)₄-TK-sAP reporter plasmid, 0.2 μ g/well of a RSV- β -lac plasmid and 0 μ g/well (black bars), 0.2 μ g/well (hatched bars), 0.5 μ g/well (open bars) or 1.0 μ g/well (shaded bars) of the indicated IRAK-1 constructs. After 24 h, cells were washed, and the sAP and β -lac activity in the media was assessed 30 h later. Results are expressed as sAP activity, normalized for β -lac expression. Data represent means $\pm$ S.E.M. for at least four independent experiments.

RESULTS

Integrity of the IRAK-1 molecule (residues 1–712) is required for overexpression-induced NF- κ B activation in unstimulated cells

Consideration of secondary structure predicts three distinct domains within IRAK-1: an N-terminal region containing a death domain, a central kinase domain containing 15 conserved residues required for ATP binding and catalytic activity [32,33] and an extensive C-terminal domain, which is absent in the *Drosophila* Pelle protein kinase and the other IRAK family members IRAK2 and IRAK-M [34] and may contain a TRAF6-binding motif [35]. We generated a set of constructs to express these domains of IRAK-1 either alone or in combination (Figure 1). Transfection of HeLa cells with increasing amounts of a plasmid expressing full-length wild-type IRAK-1 [FL-IRAK-1(1–712)], caused a 3–11-fold increase in the activation of a co-transfected NF- κ B-responsive reporter construct (Figure 2). In contrast, none of the IRAK-1 variants, when overexpressed, had any effects on NF- κ B activity in the absence of cytokine

stimulation (Figure 2). These results suggest that all three regions of the IRAK-1 molecule are essential for its ability to initiate signal transduction in a cytokine-independent manner. This is consistent with previously published data [21,36].

To confirm that all the IRAK-1 variants were expressed in HeLa cells, Western blots of cell extracts were probed with either an anti-IRAK antibody [IRAK-1-FL(1–712), IRAK-1-KC(212–712), IRAK-1-NC(1–211:533–712) and IRAK-1-CT(533–712)] or an anti-M2 FLAG antibody [IRAK-1-NT(1–211), IRAK-1-NK(1–532) and IRAK-1-K(212–532)] (Figures 3A–3G). Immunoreactive bands of the predicted size for each of the variant forms of IRAK-1 were detected (Figures 3A–3G). Endogenous IRAK-1 was detected as a 76 kDa band using the anti-IRAK antibody (lane 1 of Figures 3A and 3E). Two bands, unrelated to IRAK-1, of approx. 23 kDa and 28 kDa (lane 1 of Figure 3C), were always detected when using the anti-M2 FLAG antibody. In the absence of cytokine stimulation, the IRAK-1-FL(1–712), IRAK-1-NC(1–211:533–712) and IRAK-1-NK(1–532) constructs showed heterogeneity on Western blots, producing slower-migrating forms of IRAK-1. Treatment of the cell extracts with a protein phosphatase, PP-2A₁ (for example, see Figure 3H), resulted in only the faster-migrating band being detected, indicating that these slower-migrating forms were due to IRAK-1 phosphorylation on serine/threonine residues.

Full-length IRAK-1 and an N-terminal domain truncated version of IRAK [IRAK-1-KC(212–712)] both activated NF- κ B upon cytokine stimulation

As the mutant IRAK-1 constructs did not exhibit any effects on NF- κ B activation under non-stimulated conditions, their effects on cytokine-stimulated NF- κ B activation were investigated. HeLa cells transfected with reporter genes alone responded to IL-1 β (10 pM) and TNF α (15 ng/ml) stimulation, giving 10-fold and 11-fold increases in sAP production respectively. As expected, expression of full-length WT IRAK-1 dose-dependently potentiated NF- κ B-dependent reporter gene activity following cytokine stimulation (Figure 4A). At the maximum dose of WT IRAK-1 tested (1 μ g), reporter gene activity increased to approx. 250 and 207% of the mock levels following IL-1 β and TNF α stimulation respectively (Figure 4A). As expected, the immunostaining of the slower-migrating band, identified as phosphorylated full-length IRAK-1, was increased following IL-1 β stimulation (Figure 3A). In contrast, phosphorylation levels were markedly decreased following addition of TNF α (Figure 3A). The IRAK-1 construct expressing the kinase and C-terminal domains of IRAK, IRAK-1-KC(212–712), caused a similar potentiation of cytokine-induced NF- κ B activation, although to a lesser extent than the full-length IRAK-1 protein (Figure 4A). Unlike full-length IRAK-1, however, neither IL-1 β nor TNF α induced the phosphorylation of this construct (Figure 3B).

Expression of specific domains or truncated variants of IRAK-1 inhibited NF- κ B activation following cytokine stimulation

The constructs expressing either the N-terminal domain of IRAK-1, IRAK-1-NT(1–211), or the C-terminal domain of IRAK-1, IRAK-1-CT(533–712), acted as inhibitors of both IL-1 β - and TNF α -induced NF- κ B reporter gene activity (Figure 4B). Alkaline phosphatase reporter gene levels were inhibited by IRAK-1-NT(1–211) following IL-1 β and TNF α stimulation, by 49 $\pm$ 8.7 and 56.4 $\pm$ 12.4% respectively (Figure 4B). Similar results were seen on overexpression of the C-terminal domain construct, where both IL-1 β - and TNF α -stimulated NF- κ B activity was inhibited by approx. 60% (Figure 4B). Neither construct displayed any signs of phosphorylation following

Figure 3 Expression and phosphorylation of the IRAK-1 variants in the presence and absence of cytokine stimulation

(A–G) Western blots of whole-cell extracts from transfected HeLa cells were probed with an anti-IRAK antibody [IRAK-1-FL(1–712), IRAK-1-KC(212–712), IRAK-1-CT(533–712) and IRAK-1-NC(1–211:533–712)] or an anti-FLAG antibody [IRAK-1-NT(1–211), IRAK-1-NK(1–532) and IRAK-1-K(212–532)]. Lane 1, empty pBKRVS vector; lanes 2–4, 0.2 μ g, 0.5 μ g and 1.0 μ g of the indicated IRAK-1 constructs following stimulation with 10 pM IL-1 β or 15 ng/ml TNF α respectively. Results shown are representative of those from at least two other experiments. (H) A whole-cell extract from HeLa cells expressing the IRAK-1-NC(1–211:533–712) was treated for indicated times with protein phosphatase-2A prior to Western blotting and detection with anti-IRAK antibody. Similar results were obtained for each construct where heterogeneity was observed.

Figure 4 Effects of full-length IRAK-1 and the IRAK-1 variants on cytokine-stimulated NF- κ B reporter gene activity

HeLa cells (12-well plates) were co-transfected with 0.3 μ g/well of an (NF- κ B) $_L$ -TK-sAP reporter plasmid, 0.2 μ g/well of an RSV- β -lac plasmid and 0 μ g/well (black bars), 0.2 μ g/well (hatched bars), 0.5 μ g/well (open bars) or 1.0 μ g/well (shaded bars) of the indicated IRAK-1 constructs. After 24 h, cells were washed, then stimulated with 10 pM IL-1 β (upper panels) or 15 ng/ml TNF α (lower panels) for 30 h. The sAP activity in the media was assessed, normalized for β -lac expression and expressed as a percentage of the ligand-induced activity in the absence of transfected IRAK-1 protein. Typically, IL-1 β and TNF α induced 10-fold and 11-fold increases in sAP production respectively in HeLa cells transfected with the reporter genes alone. Data represent the means $\pm$ S.E.M. for at least four independent experiments. (A) IRAK-1-FL(1–712) and IRAK-1-KC(212–712); (B) IRAK-1-NT(1–211), IRAK-1-CT(533–712), IRAK-1-NC(1–211:533–712), IRAK-1-K(212–532) and IRAK-1-NK(1–532)

cytokine stimulation (Figures 3C–3D). Expression of the IRAK-1-NC(1–211:533–712) construct, which consists of the N-terminal and C-terminal domains of IRAK-1 fused together without the kinase domain, also inhibited both IL-1 β - and TNF α -stimulated NF- κ B reporter gene activity (Figure 4B). Like the full-length construct, phosphorylation of IRAK-1-NC(1–211:533–712) was increased following IL-1 β stimulation, but not on addition of TNF α (Figure 4E). Expression of the kinase domain of IRAK-1 alone, IRAK-1-K(212–532), had no effect on IL-1 β - or TNF α -driven activation of NF- κ B and it did not become phosphorylated upon cytokine stimulation (Figures 3F and 4B).

Expression of a C-terminal truncated version of IRAK-1, IRAK-1-NK(1–532), also inhibited IL-1 β - and TNF α -stimulated NF- κ B-reporter activity (Figure 4B). Inhibition of the IL-1 β response was observed at the lowest plasmid concentration tested (30% at 0.2 μ g), reaching an inhibitory effect of 70% at the highest dose tested (Figure 4B). Significant inhibition of TNF α -driven NF- κ B-reporter activity was also observed at the highest IRAK-1-NK(1–532) dose tested (47% at 1 μ g) (Figure 4B). IRAK-1-NK(1–532) phosphorylation was not increased upon IL-1 β stimulation, although the levels of phosphorylation observed were decreased upon stimulation with TNF α , as seen with the full-length protein (Figure 3G).

Migration of IRAK-1 into the IKK complex following IL-1 β stimulation

To try to relate these observations to interactions of IRAK-1 with other signalling proteins implicated in NF- κ B activation, we established methods to examine endogenous IRAK-1 complexes in HeLa cells before and after stimulation with IL-1. To

Figure 5 Gel-filtration analysis of endogenous IRAK-1 in HeLa cells

HeLa cell extracts were prepared from unstimulated cells or from cells that had been treated with IL-1 β for 5 min as described in the Experimental section and separated on a Sepharose Superdex 200HR column (Pharmacia). Fractions were analysed using Western blotting with anti-IRAK-1 or anti-NEMO antibodies. The molecular sizes represented in each fraction were calculated following calibration of the column with Bio-Rad gel-filtration standards. Results shown are representative of those from at least two other independent experiments. The exposure times of all blots were the same. The variation seen between the two halves of Figure 5(C) is simply due to differences in background arising from normal variation in the processing of different blots.

investigate the biochemical characteristics of native and activated IRAK-1 complexes, HeLa cell extracts were made before and following 5 min IL-1 β stimulation and the soluble cell fractions subjected to size-exclusion chromatography on a Superdex 200HR gel-filtration column. Identification of IRAK-1, NEMO and IKK β in individual fractions was achieved by SDS/PAGE analysis and by probing the Western blots with specific antibodies

Figure 6 Endogenous IRAK-1 and NEMO co-immunoprecipitate upon IL-1 β stimulation

HeLa-cell cytoplasmic extracts were prepared from control cells and cells treated with 50 pM IL-1 β for 5 min as described in the Experimental section. Immunoprecipitation (IP) was performed using an anti-NEMO antibody. Samples were separated using 8% polyacrylamide/Tris/glycine gels and immunoblotted with anti-IRAK-1 (A), anti-IKK β (B) or anti-NEMO (C) antibodies, in the presence or absence of a competing IRAK peptide, which blocked binding of the IRAK antibody.

(Figure 5). As previously reported [13,14], NEMO was eluted in fractions corresponding to a high-molecular-mass complex (600–900 kDa) equivalent to that noted by others for the IKK–signalsome complex (Figure 5A) [12]. Furthermore, this peak corresponded precisely with the presence of IKK β (results not shown). NEMO immunoreactivity could also be observed being eluted in fractions corresponding in size to a possible NEMO homodimer (96 kDa) (Figure 5A).

In resting cells, endogenous IRAK-1 immunoreactivity was eluted at a molecular size consistent with the existence of the inactive IRAK-1 protein as a homodimer (Figure 5B). However, the possibility of a small complex containing other components cannot be discounted. The IRAK-1 immunoreactive bands seen in these fractions correspond to the native unphosphorylated form of the protein (76 kDa). Following 5 min of IL-1 β stimulation, there was a marked shift in the elution profile of IRAK-1 complexes on gel filtration, as well as changes in the patterns of IRAK-1 immunoreactivity seen on Western blots (Figure 5C). IRAK-1, like NEMO and IKK β was observed in column fractions containing protein complexes of 600–900 kDa (Figures 5A and 5C). Moreover, Western blotting revealed multiple immunoreactive high-molecular-mass forms of IRAK-1 up to 150 kDa and produced a ‘ladder’-like appearance of the IRAK-1 protein, a phenomenon that was not observed in unstimulated cells (Figure 5B). In addition, the appearance of low-molecular-mass immunoreactive bands was observed in extracts from

activated cells, which may correspond to IRAK-1 degradation products (Figure 5C).

Following IL-1 β stimulation, IRAK-1 interacts with NEMO allowing it to be recruited to the IKK complex

To establish whether IRAK-1 is associated with the IKK complex, we determined whether the endogenous IRAK-1 protein could interact with NEMO using immunoprecipitation experiments. Anti-NEMO immunoprecipitates were prepared from control cells and cells stimulated with IL-1 β for 5 min, and blots were probed with anti-IRAK and anti-IKK β antibodies in the absence and presence of the IRAK-antigen peptide (Glu-Gln-Asp-Arg-Gln-Gly-Pro-Glu-Glu-Ser-Asp-Glu-Phe-Gln-Ser) to discriminate IRAK-1-specific antibody binding (Figures 6A and 6B). As shown in Figure 6, a band corresponding to the endogenous 76 kDa form of IRAK-1 was found to co-immunoprecipitate with NEMO only upon IL-1 β stimulation (Figure 6A). This band was absent in the immunoprecipitate from the peptide antibody preadsorbed control confirming its identity as IRAK-1. As expected, the anti-NEMO antibody also co-immunoprecipitated IKK β , both in the absence and presence of cytokine stimulation, and blots probed with anti-NEMO indicated equal immunoprecipitation efficiency under the different conditions (Figures 6B and 6C).

DISCUSSION

To investigate the functional roles of the different structural domains present within the IRAK-1 molecule, we have expressed discrete regions of this molecule and have assessed the effects on IL-1 β signalling and NF- κ B activation. Overexpression of wild-type IRAK-1 potently activates NF- κ B-dependent reporter gene expression, an effect that is independent of IL-1 β stimulation [36]. Although IRAK-1 possesses intrinsic kinase activity, mutations that eliminate this function do not affect the ability of IRAK-1 to mediate IL-1 β signal transduction [36–38]. Moreover, the IRAK-1-related proteins IRAK-2 and IRAK-M are unlikely to be catalytically active, as they lack key catalytic residues in their kinase domains. Thus it is possible that members of this family of proteins function as adapter molecules rather than as kinase-dependent signal transducers. Indeed, our studies have revealed that the kinase domain, but not the kinase activity, of IRAK-1 is essential for signalling to NF- κ B. Thus, in contrast with the full-length IRAK-1 and the IRAK-1-KC(212–712) construct, the IRAK-1 variant lacking the kinase domain, IRAK-1-NC(1–211:533–712), failed to potentiate cytokine-induced NF- κ B-dependent reporter gene activity (Figures 2, 4A and 4B). Since the kinase domain expressed on its own failed to inhibit or induce NF- κ B activation (Figure 4B), it seems unlikely that this domain of IRAK-1 can interact with other NF- κ B pathway signalling components. Rather, it is possible that ATP binding via this domain may regulate the interactions of the other domains of the protein with important components of the signalling pathway.

Overexpression of the N-terminal 211 amino acids of IRAK-1 inhibits IL-1 β -induced NF- κ B-dependent reporter gene activity (Figure 4B) [21,32,36]. This inhibitory effect is thought to be due to binding of IRAK-1-NT(1–211) to the death domain of the adapter protein MyD88, preventing access of endogenous IRAK-1 to MyD88 and the IL-1RI complex. Overexpression of the C-terminal domain (533–712) of IRAK-1 also led to dose-dependent inhibition of NF- κ B activation in response to IL-1 β (Figure 4B), suggesting that the C-terminal domain of IRAK-1 may also interact with proteins involved in this signalling pathway.

Neither domain became phosphorylated when overexpressed or in response to cytokine stimulation (Figures 3C–3D). However, the fusion protein of the two domains, IRAK-1-NC-(1–211:533–712) did show signs of phosphorylation upon overexpression, with the level of phosphorylation being increased in response to IL-1 β stimulation (Figure 3E). IRAK-1-NK(1–532) exhibited phosphorylation upon overexpression, but no increase in the level of phosphorylation was observed following treatment with IL-1 β (Figure 3G). Thus while the N-terminal domain of IRAK-1 is primarily responsible for recruiting IRAK-1 to the IL-1RI complex [21,23], the C-terminal domain is either the substrate which becomes phosphorylated in response to IL-1 β stimulation, or may be responsible for recruiting a kinase which phosphorylates the N-terminus of IRAK-1. This could be either endogenous IRAK-1 or another kinase. The IRAK-1-KC(212–712) construct was able to support NF- κ B activation (Figure 4A), but was not phosphorylated in response to cytokine stimulation (Figure 3B), suggesting that phosphorylation of IRAK-1 is not essential for signal transduction to NF- κ B.

In resting cells we have demonstrated that IRAK-1 is eluted predominantly in a complex of 150–200 kDa, with no evidence of the monomeric 76 kDa form of this protein (Figure 5B), suggesting that IRAK-1 is maintained in an inactive, unphosphorylated form as a dimer or in a small complex with other proteins. Upon IL-1 β stimulation, endogenous IRAK-1 was eluted in molecular-mass fractions of 600–800 kDa, where it could be detected along with NEMO, an integral component of the IKK complex (Figures 5A and 5C) [13–15]. Furthermore, following IL-1 β stimulation, endogenous IRAK-1 could be co-immunoprecipitated with NEMO (Figure 6). IRAK-1 has previously been shown to associate with TRAF6 following IL-1 β stimulation [24], and this may be mediated by the C-terminal domain [35]. Upon IL-1 β stimulation of 293IL-1RI cells, endogenous TRAF6 has been shown to co-immunoprecipitate with TGF β -activated kinase 1 (TAK-1) [39], and TAK-1 interacts with IKK α and IKK β , activating the IKK complex, leading to NF- κ B activation [40]. It is therefore possible that TAK-1 serves as a docking site for TRAF6 and perhaps IRAK-1, recruiting these signalling molecules into the IKK complex.

The TNF receptor-associated adapter protein RIP binds to NEMO upon TNF α stimulation, thus recruiting NEMO and the IKKs to the p55 TNF receptor complex [29]. RIP undergoes covalent modification following TNF α stimulation, suggesting that it may become ubiquitinated [29]. Following IL-1 β stimulation, we have observed a similar 'ladder'-like appearance of the IRAK-1 protein in higher-molecular-mass fractions (Figure 5), which may indicate hyperphosphorylated and ubiquitinated IRAK-1. It is possible, then, that NEMO may play a similar role in IL-1 β - and TNF α -mediated signal transduction, recruiting an adapter (IRAK-1 or RIP respectively) into the IKK complex and that IRAK-1 may act like RIP, recruiting the IKK-signalsome into the IL-1RI complex.

The work of Yamin and Miller [23] has suggested that the ubiquitination of IRAK-1 occurs following its recruitment to the IL-1RI complex. The role of the phosphorylation and ubiquitination of IRAK-1 that occurs following its recruitment to the receptor complex is unclear. It may be responsible for severing the association of IRAK-1 with the receptor complex, or may perhaps allow IRAK-1 to bind to molecules required for transmission and/or termination of IL-1-induced signals. Ubiquitination may also play a regulatory role in IKK activation [41]. It appears that TRAF6 functions as a ubiquitin ligase (E3), mediating the assembly of K63-linked polyubiquitin chains, which are linked to a target protein that remains to be identified, but is not NEMO, IKK α or IKK β [41]. Since IRAK-1 interacts

with TRAF6, appears to become ubiquitinated and is involved in activation of the IKK complex, it may be that IRAK-1 is a target protein of the K63-linked ubiquitin chains and that ubiquitination of IRAK-1 may be necessary for IKK activation.

In our experiments, the IRAK-1 constructs also exhibited effects on TNF α -driven NF- κ B activation (Figures 4A and 4B). In support of our findings, studies in IRAK-1-deficient mouse embryonic fibroblasts have shown that IL-1 β is completely unable to activate NF- κ B, whereas these cells retain some ability to respond to TNF α , although the intensity of the NF- κ B response is markedly attenuated compared with controls [42]. These results suggest that IRAK-1 is an essential component of the IL-1 β signalling pathway, but may also play some role in TNF α signal transduction to NF- κ B. Other studies have indicated that the mouse homologue of human IRAK-1 (mouse Pelle-like protein kinase) associates with the TNFR1 and is involved in a novel TRADD- and TRAF2-independent signal-transduction pathway [43]. Interestingly, while IL-1 β induces the rapid phosphorylation of full-length IRAK-1, TNF α has the opposite effect, reducing IRAK-1 phosphorylation (Figure 3A). Furthermore, IRAK-1-NK(1–532) exhibited phosphorylation upon overexpression, but no increase in the level of phosphorylation was observed following treatment with IL-1 β , whereas phosphorylation of this construct was reduced by TNF α treatment (Figure 3G), suggesting that the residues dephosphorylated in response to TNF α are distinct from those phosphorylated in response to IL-1 β . These data suggest that the involvement of IRAK-1 in the TNF signalling pathway is distinct from its effects on IL-1 signalling.

TNF α has also been shown to activate endogenous TAK-1 [40], so it is conceivable that the inhibitory effect that the C-terminal domain of IRAK-1 exerted on IL-1 β - and TNF α -induced NF- κ B activation (Figure 4B) could be due to sequestration of TRAF6 and/or TAK-1, interfering with downstream signalling events. Additionally, it is possible that IRAK-1 and RIP compete for binding to NEMO, thus explaining the inhibitory effects that the IRAK-1 variants exhibited on TNF α -stimulated NF- κ B activation.

In summary, we have used a combination of biochemical methods and overexpression studies to provide compelling evidence that IRAK-1 functions in the IL-1 β signal-transduction pathway as an adapter molecule, directly linking events at the IL-1 receptor to the cytoplasmic IKK complex and hence to NF- κ B activation. Future studies are required to define the consequences of IRAK-1 phosphorylation, and to elucidate the precise nature of the interaction of IRAK-1 with the IKK complex.

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