Mass Spectrometric Analysis of the Endogenous Type I Interleukin-1 (IL-1) Receptor Signaling Complex Formed after IL-1 Binding Identifies IL-1RAcP, MyD88, and IRAK-4 as the Stable Components*

Constantinos Brikos‡§¶, Robin Wait‡, Shajna Begum‡, Luke A. J. O'Neill§‖, and Jeremy Saklatvala‡‖

We investigated the composition of the endogenous ligand-bound type I interleukin-1 (IL-1) receptor (IL-1RI) signaling complex using immunoprecipitation and tandem mass spectrometry. Three proteins with approximate molecular masses of 60 (p60), 36 (p36), and 90 kDa (p90) became phosphorylated after treatment with IL-1. Phosphorylation *in vitro* of p60 has been reported previously, but its identity was unknown. We showed using tandem mass spectrometry that p60 is identical to interleukin-1 receptor-associated kinase (IRAK)-4. MS also enabled detection of IL-1, IL-1RI, IL-1 receptor accessory protein (IL-1RAcP), and myeloid differentiation primary response protein 88 (MyD88) in the complex. The p60 protein (IRAK-4) was the earliest component of the complex to be phosphorylated. Phosphorylated IRAK-4 from the receptor complex migrated more slowly in SDS-PAGE than its unphosphorylated form as did recombinant IRAK-4 autophosphorylated *in vitro*. Phosphorylation was restricted to serine and threonine residues. IRAK-4, p36, IL-1RAcP, and MyD88 bound to the liganded receptor within 15 s of activation by IL-1 and remained associated upon prolonged activation, suggesting that the signaling complex is very stable. The p90 phosphoprotein was only transiently associated with the receptor. This behavior and its size were consistent with it being IRAK-1. Our work revealed that liganding of IL-1RI causes its strong and stable association with IL-1RAcP, MyD88, and the previously unidentified protein p60 (IRAK-4). The only component of the IL-1RI signaling complex that dissociated is IRAK-1. Our study is therefore the first detailed description of the endogenous IL-1RI complex. *Molecular & Cellular Proteomics 6:1551–1559, 2007.*

Interleukin-1 (IL-1)[1] is a proinflammatory cytokine, the local and systemic effects of which bridge the innate and adaptive immune systems (1). There are two active IL-1 molecules: IL-1α and IL-1β with similar three-dimensional structures (2, 3) that induce similar biological responses via a common plasma membrane receptor, the type I IL-1 receptor (IL-1RI) (4–6). Association of IL-1 with its receptor results in activation of the nuclear factor κB (NF-κB) pathway (7) and the three MAPK pathways p38, p42/p44, and JNK (8). When IL-1 binds to its receptor, a signaling complex is formed, but the precise composition of this complex and how it activates downstream pathways are uncertain. Several proteins associate with the IL-1R1, including its accessory protein (IL-1RAcP) (9–11), the myeloid differentiation primary response protein 88 (MyD88) (12–14), Toll-interacting protein (Tollip) (15), and the interleukin-1 receptor-associated kinases (IRAKs). Four of the latter have been identified: IRAK-1, IRAK-2, IRAK-M, and IRAK-4 (16–19). IRAK-1 is transiently recruited to the complex via binding to MyD88 (12–14) or Tollip (15) and also associates with and activates tumor necrosis factor receptor-associated factor 6 (TRAF6) (20). It was originally thought that TRAF6 binds tightly to IRAK-1 but does not associate with the receptor signaling (20, 21). However, more recently it has been detected in the complex (22).

When IRAK-1 is phosphorylated, it leaves the receptor complex together with TRAF6, and both are recruited to a plasma membrane-associated protein complex, which contains the transforming growth factor-β-activated kinase (TAK1) and its binding partners TAB1 and TAB2 (23, 24). After phosphorylation of TAK1 and TAB2, the TRAF6-TAK1-TAB1-

From the ‡Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College London, London W6 8LH, United Kingdom and the §School of Biochemistry and Immunology, Trinity College Dublin, Dublin D2, Ireland

Received, December 4, 2006, and in revised form, April 30, 2007
Published, MCP Papers in Press, May 15, 2007, DOI 10.1074/mcp.M600455-MCP200

[1] The abbreviations used are: IL-1, interleukin-1; IL-1RI, type I interleukin-1 receptor; IL-1RAcP, type I interleukin-1 receptor accessory protein; IRAK, interleukin-1 receptor-associated kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; TAB, TAK1-binding protein; TAK1, transforming growth factor-β-activated kinase 1; Tollip, Toll-interacting protein; TRAF6, tumor necrosis factor receptor-associated factor 6; JNK, c-Jun N-terminal kinase; MyD88, myeloid differentiation primary response protein 88; DMP, dimethyl pimelimidate.

TAB2 complex translocates to the cytoplasm where TAK1 is activated (25–27) and dissociates from the complex, activating the IκB kinase (22). The IκB kinase complex phosphorylates the inhibitory proteins of NF-κB, IκBα and IκBβ, resulting in their degradation and the release of NF-κB (28–30). TAK1 also activates p38 and JNK (22, 31, 32).

Investigation of the formation and regulation of the endogenous IL-1RI complex has hitherto used immunological detection of components. These studies suggested that MyD88 associates with the receptor and may recruit IRAK-4, which can phosphorylate IRAK-1 *in vitro*. Tollip has also been detected in the complex, but its function is uncertain. Other possible components include IRAK-2, IRAK-M, and Pellino, although none have been detected in the endogenous complex. Uncertainties about the catalytic activity of IRAK-1 and IRAK-4 remain. Overexpression of IRAK-1 results in activation of downstream signaling (33). Mutant cells lacking IRAK-1 are unresponsive to IL-1 (34), and IRAK-1 knock-out mice have impaired *in vivo* responses to IL-1 (35). However, the protein kinase activity of IRAK-1 is not necessary for IL-1RI signaling (34, 36). Although it was initially suggested that IRAK-1 in the IL-1RI signaling complex could be autophosphorylated (16, 36), kinase-dead IRAK-1 transfected into IRAK-1$^{-/-}$ cells still becomes phosphorylated, suggesting involvement of another kinase (34). When IRAK-4 was discovered it seemed a likely candidate for the missing kinase, although this remains to be proved. IL-1 responsiveness is lost in IRAK-4 null mice (37) and in humans with IRAK-4 deficiency (38). Furthermore recombinant IRAK-4 can phosphorylate kinase-dead IRAK-1 *in vitro* (19), and IL-1-induced phosphorylation of IRAK-1 does not occur in IRAK-4-deficient cells (38) or in cells overexpressing kinase-dead IRAK-4 (19).

The first kinase activity that was observed to be associated with activated IL-1RI was due to a serine/threonine protein kinase (39) that co-precipitated with the receptor and *in vitro* was able to phosphorylate an unidentified 60-kDa protein (p60) in the complex. In the present study we used mass spectrometry to characterize components of the immunoprecipitated endogenous IL-1RI signaling complex that can be phosphorylated *in vitro*. We provide the first description of the endogenous liganded IL-1RI complex, demonstrating that IL-1RAcP and MyD88 are stably associated with the signaling complex. In addition, we identify p60 as IRAK-4 and show that it is also a stable component of the endogenous IL-1RI signaling complex.

EXPERIMENTAL PROCEDURES

Biological Reagents and Cell Culture—Recombinant human IL-1α was made by standard methods. Anti-mouse IL-1RI (M5) rat monoclonal antibody was a gift from Dr. John Sims (Amgen Corp., Seattle, WA). Anti-MyD88 rabbit antiserum was a gift from Dr. Jürg Tschopp (University of Lausanne, BIL Biomedical Research Center, Epalinges, Switzerland). Rabbit polyclonal anti-IL-1RAcP and mouse monoclonal anti-IRAK-1 antibodies were purchased from QED Bioscience Inc. and Santa Cruz Biotechnology Inc., respectively. His-tagged recombinant IRAK-4 was a gift from Dr. Holger Wesche (Amgen, South San Francisco, CA). EL4 6.1 murine thymoma cells (40) were obtained from Dr. John Sims and were grown in RPMI 1640 medium with L-glutamine (BioWhittaker, Veviers, Belgium) supplemented with 5% (v/v) FCS and 1% penicillin/streptomycin (100 units/100 mg/ml) (BioWhittaker). The cells were passaged every 1–2 days to maintain a cell density of 2×10^5–10^6 cells/ml. Large volume cell cultures (500–1500 ml) were grown in spinner flasks (Techne, Cambridge, UK). These cultures were supplied with 5% (v/v) CO_2, sealed, and incubated at 37 °C with gentle stirring.

Stimulation and Lysis of EL4 6.1—EL4 6.1 cells were stimulated with recombinant human IL-1α (20 ng/ml) at a density of 10^7 cells/ml at 37 °C. For large scale experiments, cells were stimulated for 5 min, placed on ice, and washed three times in ice-cold PBS. Ice-cold lysis buffer (1% Brij96, 50 mM NaCl, 50 mM Tris, pH 7.4, 2 mM PMSF, 1 μg/ml pepstatin, 10 μM E64, 15 μg/ml aprotinin) was added to the cell pellets (125 μl/10^7 cells), which were immediately vortexed and left on ice for 30 min. For small scale experiments (5×10^7 or 10^8 cells per point), 1 volume of 2× ice-cold lysis buffer (2% Brij96, 100 mM NaCl, 100 mM Tris, pH 7.4, 4 mM PMSF, 2 μg/ml pepstatin, 20 μM E64, 30 μg/ml aprotinin) was added directly to the stimulated cultures, which were vortexed and left on ice for 30 min.

Preclearing of the Lysates and Immunoprecipitation of the IL-1RI Signaling Complex—Insoluble cellular debris and nuclei were removed from the lysates by centrifugation (13,000 rpm for 20 min at 4 °C in a microcentrifuge). For some small scale experiments (5×10^7 cells per point), the supernatants were precleared with rat IgG (Sigma reagent grade) and protein G-Sepharose beads (Amersham Biosciences) prior to immunoprecipitation. 50 μg of purified rat IgG (Sigma reagent grade) were added to the supernatants, and they were rotated for 1 h at 4 °C. Subsequently 50 μl of settled protein G-Sepharose beads were added, and the mixtures were rotated for 3–4 h. For Western blotting of immunoprecipitates the rat IgG was cross-linked to the protein G-Sepharose beads (see below). For large scale experiments, preclearing was performed with disposable polystyrene columns (Pierce) containing rat IgG cross-linked to the protein G-Sepharose beads. Lysates from 1.5×10^9 cells (after the removal of their nuclei and cellular debris) were passed through a 1-ml column at 4 °C.

The IL-1RI in the precleared solubilized membrane preparation was immunoprecipitated with either 1 or 5 μg (small scale) or 20 (large scale) μg of M5 antibody bound to 40–50 μl of protein G beads. The mixtures were rotated for 4 h at 4 °C. In some experiments, the antibody was first added to the precleared lysates, the samples were rotated at 4 °C for 1 h, and then each sample was rotated for another 3–4 h after the beads were added. Alternatively precoated beads with M5 antibody (by cross-linking) were added to the precleared lysates, and the mixtures were rotated for 4 h at 4 °C.

Cross-linking of the Rat IgG or M5 Antibody to Protein G-Sepharose Beads—Cross-linking was performed with dimethyl pimelimidate (DMP; Sigma). The beads were washed three times with 0.2 M triethanolamine, pH 9.0. For cross-linking rat IgG to protein G-Sepharose beads, the IgG, beads, and DMP (at a ratio of 1 μg of IgG/0.5 or 1 μl of settled protein G-Sepharose slurry/50 μg of DMP) were rotated for 1 h at room temperature in 0.2 M triethanolamine, pH 9.0 (10–15 μl of triethanolamine solution/1 μl of settled protein G-Sepharose slurry). The beads were washed twice with triethanolamine, rotated in fresh solution for 2 h at room temperature, and then washed with PBS and stored at 4 °C. Coated beads were washed with lysis buffer before use. For cross-linking M5 or rat IgG to protein G-Sepharose more DMP was used as specified in the figure legends.

In Vitro Phosphorylation of Immunoprecipitated IL-1RI Complexes and Recombinant IRAK-4—The immunoprecipitated IL-1RI complexes were washed four times with lysis buffer containing 1% Brij96,

50, 500, or 1000 mM NaCl, 50 mM Tris, pH 7.4, and twice with kinase buffer (20 mM HEPES, pH 6.5, 100 mM NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$). *In vitro* phosphorylation was performed on the beads at room temperature for 30 min. Each reaction mixture contained 8.5 μCi of [γ-^{32}P]ATP in the case of small scale experiments or 10 μCi [γ-^{33}P]ATP (Amersham Biosciences) in the case of large scale experiments. Each reaction also contained non-radioactive ATP at a final concentration of 1 μM. In the large scale experiments, after phosphorylation the immunoprecipitates were washed once with 1 ml of radioimmune precipitation assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM sodium fluoride, 25 mM β-glycerophosphate, 10 mM tetrasodium pyrophosphate, 1 mM sodium orthovanadate, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) and once with 50 mM Tris, pH 7.4. 3 volumes of SDS sample buffer were added to the mixtures, which were subsequently heated at 95–100 °C for 3–5 min and loaded onto SDS-polyacrylamide gels. The *in vitro* autophosphorylation of recombinant IRAK-4 was performed at room temperature for 150 min in 40 μl of kinase buffer containing 20 μM ATP and 1 μCi of [γ-^{32}P]ATP.

Phosphoamino Acid Analysis—The radiolabeled bands were in-gel digested with trypsin, and the resulting peptides were hydrolyzed for 1–3 h at 110 °C in 250 μl of 50% (v/v) HCl (~5.7 M). Phosphoamino acid analysis was carried out using a Hunter apparatus (41). The sample was resuspended in 10 μl of electrophoresis buffer (7.8% (v/v) acetic acid, 2.2% formic acid, pH 1.9) and spotted onto a thin layer chromatography cellulose plate (Anachem Ltd., Beds, UK). Phosphotyrosine, phosphothreonine, and phosphoserine standards (2 μg each) were also spotted in the same location. The plate was equilibrated with electrophoresis buffer and electrophoresed in the Hunter apparatus at 1.5 kV for 30 min, then dried, rehydrated in the second dimension electrophoresis buffer (0.5% (v/v) pyridine, 5% (v/v) acetic acid, pH 3.5), and electrophoresed under the same conditions at a right angle. The plate was then dried, sprayed with 5% (w/v) ninhydrin in acetone, and heated until the phosphoamino acid standards appeared. The radiolabeled amino acids were visualized by autoradiography or with a PhosphorImager.

Electrospray Ionization Mass Spectrometry—SDS-PAGE-separated proteins were visualized with mass spectrometry-compatible silver staining (42), excised, and in-gel digested using a robotic system as described previously (43). Tandem mass spectra were recorded using a Waters Q-Tof instrument (Waters, Manchester, UK) interfaced to a Waters CapLC capillary chromatograph. Samples were dissolved in 0.1% aqueous formic acid, injected onto a PepMap C18 column (300 μm× 0.5 cm; LC Packings, Amsterdam, The Netherlands), and eluted with an acetonitrile-0.1% formic acid gradient (5–70% acetonitrile over 20 min) at a flow rate of 1 μl/min. The capillary voltage was 3500 V. A survey scan over the *m/z* range 400–1300 was used to identify protonated peptides with charge states of 2, 3, or 4, which were automatically selected for data-dependent MS/MS analysis and fragmented by collision with argon. The resulting product ion spectra were transformed onto a singly charged *m/z* axis using a maximum entropy method (MaxEnt3, Waters), and proteins were identified by correlation of uninterpreted spectra to entries in Swiss-Prot/TrEMBL using ProteinLynx Global Server (Version 1.1, Waters) (44). The database was created by merging the FASTA format files of Swiss-Prot/TrEMBL and their associated splice variants (1,768,175 entries at the time of writing). No taxonomic or protein mass and pI constraints were applied. One missed cleavage per peptide was allowed, and the initial mass tolerance window was set to 100 ppm. In parallel, the spectra were also searched against the National Center for Biotechnology Information non-redundant (NCBI nr) database (4,076,784 sequences) using Mascot (Matrix Science). For an identification to be considered valid we required that two or more peptides independently matched the same protein sequence, that the peptide score was significant, typically greater than 55 ($p < 0.05$), and that manual interpretation confirmed agreement between spectra and peptide sequence. In addition Mascot searches of all spectra were performed against a randomized version of the NCBI database using the same parameters as in the main search. In no case did this search retrieve more than a single peptide, and in all instances the peptide score was below the 0.05 significance level.

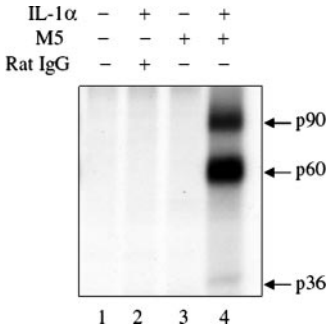

FIG. 1. **Endogenous proteins of IL-1-stimulated EL4 6.1 cells that bind to IL-1RI and become phosphorylated *in vitro*.** Precleared lysates from IL-1α-stimulated (5 min; 20 ng/ml) or unstimulated EL4 6.1 cells were used for immunoprecipitation of IL-1RI with 5 μg of M5 (anti-IL-1RI) or rat IgG (control) that were cross-linked to protein G-Sepharose beads by using 5.2 mg of DMP. The immunoprecipitates were subjected to *in vitro* phosphorylation using [γ-^{32}P]ATP. The reactions were stopped and run on a 12.5% polyacrylamide gel, which was dried and used for autoradiography. The figure shows the autoradiogram of the gel. The result shown is the representative of at least six independent experiments.

Western Blotting of the Immunoprecipitated IL-1RI Signaling Complex and Immunodetection of Its Components—SDS-PAGE-separated IL-1RI immunoprecipitates were transferred to PVDF membranes (DuPont) using a trans-blot chamber (Bio-Rad). The membranes were blocked with 5% (w/v) dried skimmed milk powder in PBS containing 0.05% (v/v) Tween 20 and incubated with primary antibodies at a dilution of 1:1000 for 1 h at room temperature or overnight at 4 °C. After washing in PBS containing 0.05% (v/v) Tween 20 the membranes were incubated for 1 h at room temperature with anti-rabbit or anti-mouse IgG horseradish peroxidase-conjugated pig or rabbit sera, respectively (Dako Ltd., High Wycombe, UK) diluted 1:1000 (v/v). The membranes were washed, and proteins were detected by ECL (Amersham Biosciences) according to the manufacturer's instructions. Blots were stripped and reprobed using the Re-Blot Western blot recycling kit (Chemicon International Inc., Hampshire, UK).

RESULTS

Liganded IL-1RI Signaling Complexes Contain Three Proteins That Become Phosphorylated in Vitro—To detect phosphorylatable components of the IL-1RI signaling complex, IL-1RI was immunoprecipitated from 10^8 EL4 6.1 murine thymoma cells with an antibody that binds an extracellular epitope remote from the IL-1 binding site (40). The immunoprecipitates were phosphorylated *in vitro*, separated by one-dimensional PAGE, and autoradiographed. A 60-kDa phosphorylated band (presumably identical to the p60 protein reported by Martin *et al.* (39) was detected only after IL-1 stimulation (Fig. 1, *lane 4*). Two further phosphoproteins of molecular masses of ~36 (p36) and 90 kDa (p90) were also detected.

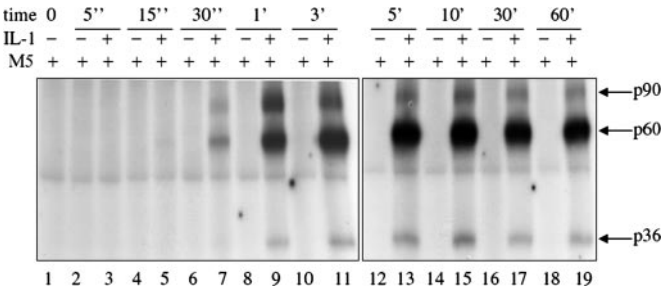

FIG. 2. ***In vitro* phosphorylation of IL-1RI immunoprecipitates from cells stimulated for different time.** 50×10^6 EL4 6.1 cells were stimulated for different periods of time with IL-1α or vehicle and used for immunoprecipitation of IL-1RI. The antibody was first added to the precleared lysates, and the samples were rotated at 4 °C for 1 h. Then the beads were added, and each sample was rotated for another 3–4 h. The immunoprecipitates were washed with lysis buffer containing 1 M NaCl and phosphorylated *in vitro*. The figure shows the autoradiogram of the dried gel and is representative of three independent experiments. p60, p90, and p36 are indicated by *arrows*. ″, seconds; ′, minutes.

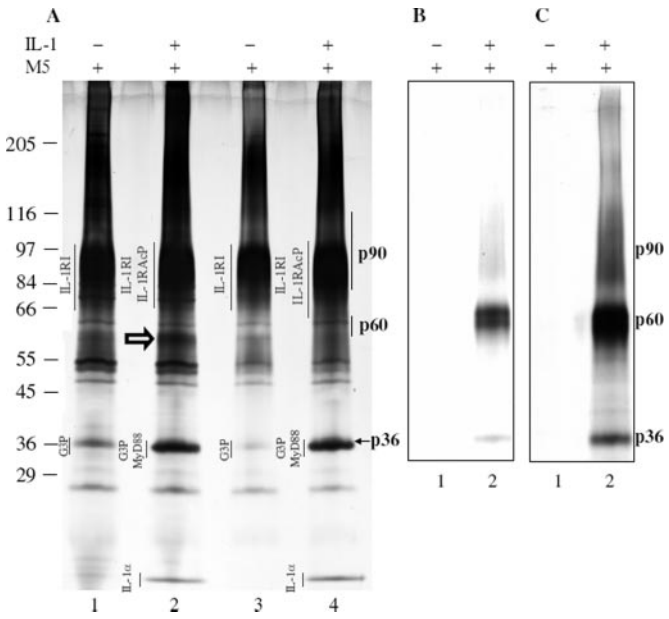

FIG. 3. **Identification of IL-1RI signaling complex components from 3 × 10^9 EL4 6.1 cells.** Precleared lysates of 3×10^9 IL-1α -stimulated (5 min; 10 ng/ml) or unstimulated cells were used for the immunoprecipitation of IL-1RI complexes. The immunoprecipitation was carried out with 20 μg of M5 cross-linked to protein G-Sepharose by using 45 mg of DMP. *A* shows the silver-stained 8–16% gradient gel. The areas of interest are indicated. *Lanes 1* and *2*, M5 immunoprecipitates from unstimulated and stimulated cells, respectively, without being used for an *in vitro* phosphorylation; *lanes 3* and *4*, immunoprecipitates from unstimulated and stimulated cells, respectively, after an *in vitro* phosphorylation using [γ-^{33}P]ATP. *B*, the autoradiogram of *lanes 3* and *4* of the gel in *A* after a ~2-h exposure at room temperature. *C*, the autoradiogram of the same part of the gel after an overnight exposure. The *arrow* indicates the silver-stained ~60-kDa protein. *G3P*, glyceraldehyde-3-phosphate dehydrogenase.

To determine the kinetics of association of these components, IL-1RI immunoprecipitates were analyzed after liganding with IL-1 for periods between 5 s and 1 h (Fig. 2). The p60 protein appeared 15 s after addition of IL-1, and its concentration was maximal between 1 and 3 min. The p90 protein was detectable at 30 s but decreased from 1 min, suggesting either a transient association or alternatively that *in vivo* phosphorylation could prevent its detection in the phosphorylation assay. The p36 protein was stably associated with the complex.

Identification of p60 as IRAK-4—Because in these experiments the phosphorylated proteins were only detectable by autoradiography and were not visible on the silver-stained gels, a larger scale immunoprecipitation, using 3×10^9 cells, was performed. Nonspecific binding was reduced by preclearing with rat IgG-protein G-Sepharose beads. The rat IgG and the M5 were cross-linked to the beads to avoid contamination of the samples with IgG. To exclude proteins that bound nonspecifically to the receptor-IgG-Sepharose complexes, the immunoprecipitates were washed with a buffer containing 0.5 M NaCl before the *in vitro* phosphorylation and with radioimmune precipitation assay buffer afterward. Phosphorylation was performed with [γ-^{33}P]ATP to improve the resolution of the autoradiograms, and polyacrylamide gradient gels were used to optimize separation of the proteins. Because phosphorylation may alter the electrophoretic mobility of proteins, immunoprecipitated complexes that had not been phosphorylated *in vitro* were run in an adjacent lane in case unphosphorylated p60 ran as a sharper and more easily detectable band.

Fig. 3*A* shows the silver-stained gel of immunoprecipitates obtained from IL-1-stimulated (for 5 min) and unstimulated cells. Stimulated cells that had not been *in vitro* phosphorylated exhibited a band of about 60 kDa (indicated by an *arrow* in Fig. 3*A*, *lane 2*), which was absent in unstimulated cells (Fig. 3*A*, *lane 1*) and which also disappeared after *in vitro* phosphorylation (Fig. 3*A*, *lane 4*). Autoradiography of the phosphorylated complexes revealed the previously observed phosphoproteins (Fig. 3, *B* and *C*, *lane 2*). Alignment of the autoradiogram with the silver-stained gel showed that p60 (Fig. 3*A*, *lane 4*) migrated more slowly and diffusely than the 60-kDa band (Fig. 3*A*, *lane 2*), and that was the reason it could not be detected by silver staining.

The 60-kDa band from the IL-1-stimulated immunoprecipitate was excised, in-gel digested with trypsin, and analyzed by tandem MS. One peptide, SITNNFDEQPASAGGNR, mapped onto a hypothetical murine protein (TrEMBL accession number Q9D250), which was similar to the N-terminal regions (residues 1–117) of two human sequences (TrEMBL accession numbers Q9Y589 and Q9NWZ3). The Q9Y589 protein subsequently proved to be identical to the then unknown IRAK-4 (19). Searching the mass spectra against the NCBI nr database once the complete sequence of murine IRAK-4 had been deposited revealed several additional IRAK-4-derived peptides, including VAQGTANGIR, SANILLDKDFTAK, and

TABLE I

Sequences of the components of the IL-1RI signaling complex identified by Q-TOF tandem mass spectrometry

IL-1RI was immunoprecipitated from precleared lysates of 3×10^9 unstimulated or IL-1α-stimulated EL4 6.1 cells. The immunoprecipitates were prepared and resolved as described in the legend of Fig. 3. The protein components of the IL-1RI immunoprecipitates were identified by Q-TOF tandem mass spectrometry as described under "Experimental Procedures." Uninterpreted MS/MS spectra were searched against the NCBI nr database (4,076,784 sequences at the time of searching). Individual ion scores greater than 55 indicate identity or extensive homology.

Protein	UniProt accession number	Peptide sequence	Mascot ion score
Human IL-1α	P01583	DDAKITVILR	46
		SAPFSFLSNVK	52
		SSKDDAKITVILR	41
		YEFILNDALNQSIIR	40
		TQLYVTAQDEDQPVLLK	109
		TITGSETNLLFFWETHGTK	81
		ANDQYLTAAALHNLDEAVK	100
		ISKTQLYVTAQDEDQPVLLK	94
Murine IL-1RI	P13504	LLTLDPVR	59
		DRPVILSPR	46
		TPISADRDSR	42
		TYDAYILYPK	22
		LLTLDPVRDTK	41
		VIQFITIDENKR	28
		NVAEEHRGDYICR	23
		DENNELPEVQWYK	46
		ASDGKTYDAYILYPK	57
		IHQQNEHLWFVPAK	63
		TLGEGSFSDLDTFVFK	126
		NCKPLLLDNVSFFGVK	66
		LHIAGDGSLVCPYVSYFK	20
		NCKPLLLDNVSFFGVK	56
Murine IL-1RAcP	Q9NPH3	QGLSYSSLK	36
		VAFPLEVVQK	43
		DSLPGGIVTDETLSFIQK	83
		LLVVLSPNYVLQGTQALLELK	45
Murine MyD88	P22366	ALSLP	18
		LSLFLNPR	42
		RLSLFLNPR	32
		VESSVPQTK	43
		ELETRPDPTR	16
		FALSLSPGVQQK	53
		FITICDYTNPCTK	72
		QQNQESEKPLQVAR	52
		LLELLALLDREDILK	89
		DVLPGTCVWSIASELIEKR	69
Murine IRAK-4	Q8R4K2	SITNNFDEQPASAGGNR	81
		VAQGTANGIR	72
		SANILLDKDFTAK	66
		ISDFGLAR	34
		LSCLDGTPPLSWHTR	40

ISDFGLAR (Table I). Several IRAK-4 sequences (Table I) were then also detected in the tryptic digest of phosphorylated p60 (Fig. 3*A*, *lane 4*). No IRAK-4-related sequences were detected in immunoprecipitates from unstimulated cells (Fig. 3*A*, *lanes 1* and *3*).

Mass spectrometric analysis of trypsin-digested gel slices enabled identification of several other components of the signaling complex. Peptides derived from IL-1RI were detected in the diffusely stained region of the gel between 70 and 100 kDa (Table I) in both unstimulated and stimulated cells (Fig. 3*A*, *lanes 1–4*). Peptides corresponding to IL-1RAcP were also present in this region of the gel but only in stimulated cells (Fig. 3*A*, *lanes 2* and *4*). As with IL-1RI, the accessory protein migrated as a diffuse smear presumably because both proteins are heterogeneously glycosylated. The accessory protein was not detected in immunoprecipitates from unstimulated cells (Fig. 3*A*, *lanes 1* and *3*), confirming that IL-1RAcP is recruited only after IL-1 binds to the receptor.

The silver-stained bands at 20 and 35 kDa in gels from stimulated cells were identified by MS as IL-1α and MyD88, respectively (Fig. 3*A*, *lanes 2* and *4*, and Table I). Peptides matching glyceraldehyde-3-phosphate dehydrogenase were also sequenced from the 35-kDa band. A silver-stained band was present at about 35 kDa in unstimulated cells (Fig. 3*A*, *lanes 1* and *3*), but only glyceraldehyde-3-phosphate dehydrogenase was detectable in this band. Phosphorylated p36 (Fig. 3, *B* and *C*, *lane 2*) overlapped with the upper part of the 35-kDa band of *lane 4* of this silver-stained gel (Fig. 3*A*).

The phosphoprotein p90 (Fig. 3, *B* and *C*, *lane 2*) lies in the region of 80–120 kDa, and its position is indicated on the silver-stained gel (Fig. 3*A*, *lane 4*). The IL-1RI and its accessory protein also migrated in this region and partially overlapped with p90.

Phosphoamino Acid Analysis of p60 and in Vitro Autophosphorylation of Recombinant IRAK-4—In the *in vitro* phosphorylated immunoprecipitates, IRAK-4 (p60) may be either phosphorylated by itself or by another component of the complex. Autophosphorylation of recombinant IRAK-4 was investigated by electrophoresis and analysis using a Phosphorimager. Coomassie staining of the gel showed that the phosphorylated form migrates more slowly than the unphosphorylated protein (Fig. 4*A*, *lanes 1* and *2*, respectively). The region of the gel shown by the Phosphorimager to be occupied by phosphorylated IRAK-4 (Fig. 4*B*, *lane 2*) is indicated by a *line* at the *right* of the Coomassie stained gel (Fig. 4*A*).

Phosphoamino acid analysis of *in vitro* phosphorylated recombinant IRAK-4 (Fig. 4, *A*, *lane 2*, and *B*, *lane 2*) detected both phosphoserine and phosphothreonine (Fig. 5*A*); the latter predominated. Similar results were obtained with *in vitro* phosphorylated p60 bound to the IL-1RI complex (Fig. 5*B*), consistent with IRAK-4 and p60 being identical.

Analysis of the IL-1 Receptor Signaling Complex by Immunoblotting—The transiently complex-associated p90 phosphoprotein ran diffusely on electrophoresis and partially comigrated with the receptor itself and the accessory protein. Phosphorylated IRAK-1 is known to migrate in the 80–100-kDa region and associates transiently with the receptor complex (15, 22, 45, 46). IRAK-1 is therefore a potential candidate for being the p90 phosphoprotein. The kinetics of IRAK-1 and

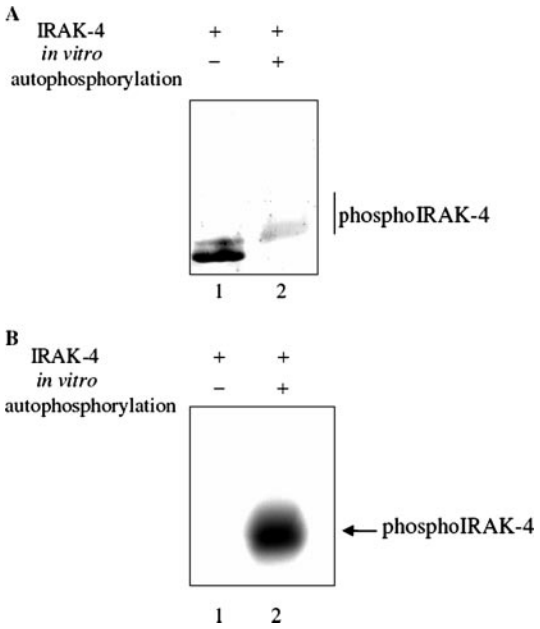

FIG. 4. ***In vitro* autophosphorylation of recombinant human IRAK-4.** ~1 μg of His-tagged human IRAK-4 was used in autophosphorylation reactions *in vitro*. The reactions were stopped by using 2× SDS sample buffer and heating them at 100 °C for 5 min and then electrophoresed on a 12.5% polyacrylamide gel. *A*, *lane 1*, IRAK-4 that was not used for *in vitro* autophosphorylation reaction (control); *lane 2*, autophosphorylated IRAK-4. *Lanes 1* and *2* are Coomassie Brilliant Blue G-stained. *B*, the phosphorimage of *A*. The *line* in *A* indicates the area of the gel that is occupied by the phosphorylated IRAK-4 according to the phosphorimage of *A*. The result shown is the representative of at least three independent experiments.

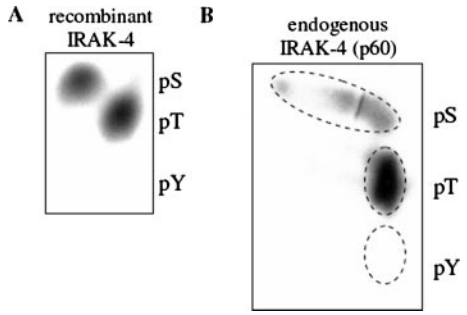

FIG. 5. **Phosphoamino acid analysis of recombinant IRAK-4 autophosphorylated *in vitro* and the IL-1 receptor-associated component p60 (endogenous IRAK-4).** Recombinant IRAK-4 was subjected to an *in vitro* autophosphorylation reaction as described in the legend of Fig. 4. Immunoprecipitates of IL-1-stimulated EL4 6.1 cells were prepared and used for an *in vitro* phosphorylation reaction as described in the legend of Fig. 1 and under "Experimental Procedures." All the phosphorylated bands were excised from their gels and used for phosphoamino acid analysis. The positions of the markers are indicated and labeled next to the phosphorimages (*pS*, phosphoserine; *pT*, phosphothreonine; *pY*, phosphotyrosine). The result shown is the representative of three independent experiments.

p90 receptor association were compared by stimulating EL4 6.1 cells for different times, immunoprecipitating IL-1RI, and immunoblotting for IRAK-1. Similarly to p90 (Fig. 2), IRAK-1

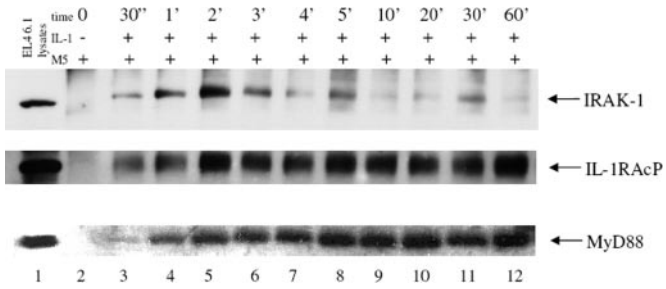

FIG. 6. **Western blotting of IRAK-1, IL-1RAcP, and MyD88 in the IL-1 receptor signaling complex from EL4 6.1 cells.** IL-1RI was immunoprecipitated from 50 × 10^6 EL4 6.1 cells that were left unstimulated (*lane 2*) or were stimulated with IL-1 for 30 s to 60 min (*lanes 3–12*). The immunoprecipitation was carried out with 1 μg of M5 cross-linked to protein G-Sepharose beads. The immunoprecipitates were washed with lysis buffer containing 50 mM NaCl, electrophoresed, and used for Western blot. Whole cell lysates of 1.5 × 10^6 unstimulated EL4 6.1 cells were used as control (*lane 1*). The membrane was stained using a mouse anti-IRAK antibody, then restained using a rabbit anti-IL-1RAcP antibody, then stripped, and restained using anti-MyD88 rabbit antiserum. The result shown is the representative of three independent experiments. ″, seconds; ′, minutes.

was detected only in stimulated cells, appearing within 30 s and reaching a maximum at 2 min before declining (Fig. 6). Mass spectrometric analysis of the IL-1RI signaling complex indicated that IL-1RAcP and MyD88 were only present in immunoprecipitates of stimulated cells (Fig. 3), and this was confirmed by immunostaining (Fig. 6).

DISCUSSION

We investigated the composition of the endogenous IL-1RI signaling complex using an analytical strategy based on immunoprecipitation and tandem MS. Our study is the first major direct analysis of the receptor signaling complex and provides a novel technical protocol that could be useful for the study of similar endogenous receptor/adapter signaling complexes like those of the Toll-like receptors, the wider family of IL-1RI. Our approach is the only one that enabled the simultaneous identification of five components of the IL-1RI signaling complex and the assessment of their possible phosphorylation. The detected proteins were IL-1, IL-1RI, IL-1RAcP, MyD88, and IRAK-4. Previously only IRAK-1 had been purified from liganded IL-1RI complex and only in a system overexpressing IL-1RI (16). One of the key findings of our work is that IRAK-4 is the unidentified protein p60 (39), and this is actually the first component of the IL-1RI signaling complex to be phosphorylated *in vitro*. We also showed the kinetics of association of the identified proteins and IRAK-1 to the liganded receptor. To our knowledge, our work is the only study that simultaneously examined the kinetics of the binding of these proteins to IL-1RI. In our system, which is not based on protein overexpressions, we demonstrated for first time, stable recruitment of the IL-1RAcP and MyD88 to the endogenous receptor upon stimulation for up to an hour with IL-1. In addition, we showed that endogenous IRAK-4 also binds

stably to the receptor signaling complex. Therefore, our work clarifies and completes the picture of the formation of the endogenous IL-1RI signaling complex by its components IL-1, IL-1RI, IL-1RAcP, MyD88, and IRAK-4.

The protein p60 has been reported in several studies since its first observation in 1994 (39), but all these years it had been one of the detected but unknown possible components of the IL-1RI signaling pathway. It had only been detected (by autoradiography) as an endogenous protein that could be immunoprecipitated with the liganded IL-1RI and was phosphorylated when the immunoprecipitates were used for *in vitro* kinase assays. Its detected mass in these reports (~60 kDa) is higher than the theoretical mass of IRAK-4 (50,872 Da) because, as our data shows, its mobility in SDS-PAGE was retarded when it is phosphorylated *in vitro*. IRAK-4 was first identified by its sequence similarity to IRAK-1 (19), but direct association with the IL-1 receptor was not demonstrated. In this work, Li *et al.* (19) overexpressed IRAK-4 and MyD88 (in an IL-1-independent system) but did not manage to co-immunoprecipitate the two proteins. However, they managed to successfully co-immunoprecipitate the overexpressed kinase-dead form of IRAK-4 (IRAK-4 KK213AA) with MyD88. The explanation could be that overexpressed IRAK-4 becomes phosphorylated and dissociates from MyD88 and IL-1RI. Such an explanation would agree with another study based on overexpression of the proteins TRAF6 and Pellino1 (47). In this study, a model was proposed in which IRAK-1, IRAK-4, TRAF6, and Pellino1 dissociate from the IL-1RI signaling complex. The above studies and the hypothesized model were contradicted by another more recent report on the mode of recruitment of IRAK-4 to the IL-1RI (48). In this study, which was also based solely on overexpression of the known components of the IL-1RI signaling complex and examination of the associations with each other independently of the binding of IL-1RI by its ligand, it was shown that MyD88 can bind kinase-active IRAK-4. Our work, under physiological conditions (*i.e.* requiring IL-1 stimulation and not based on overexpressions) clarifies that IRAK-4 and MyD88 stably associate with the receptor complex. Both proteins bound rapidly to the receptor upon IL-1 stimulation and remained associated with the complex for at least 1 h. In the signaling complex IRAK-4 (p60) was hypo- or unphosphorylated, but it became hyperphosphorylated on serines and threonines on *in vitro* phosphorylation. It is likely that co-immunoprecipitation of IRAK-4 (p60) with IL-1RI creates concentrations high enough for autophosphorylation because its electrophoretic mobility and phosphoamino acid composition were consistent with those of autophosphorylated recombinant IRAK-4.

The nature of the association of IL-1RAcP with the receptor was previously unclear. An antibody (4C5) that recognized a protein distinct from the two known IL-1 receptors blocked IL-1 binding to its active receptor IL-1RI and signaling. The protein this antibody bound was identified as the IL-1RAcP. Thus, these data suggested that IL-1RAcP and IL-1RI must therefore be either preassociated or close enough together because only in that case would the binding of the antibody to IL-1RAcP prevent the access of IL-1 to its receptor. Our results show that co-immunoprecipitation of IL-1RI and IL-1RAcP was possible only when IL-1 was bound to IL-1RI. Thus, we clarify that IL-1 is definitely required for a strong and stable interaction among IL-1RI and IL-1RAcP and for the recruitment of intracellular proteins to the receptor complex. Such a receptor/accessory protein dimerization model could be in agreement with the current theory on initiation of signaling by the related Toll-like receptors (49). According to this model, a ligand binds to its Toll-like receptor forming a stable receptor-ligand complex. Consequently conformational changes occur making possible the formation of stable receptor-receptor (or perhaps receptor-accessory protein) complexes that are able to recruit cytoplasmic proteins and signal.

As previously reported (15, 22, 38, 45, 46) IRAK-1 is phosphorylated in response to IL-1 treatment and then dissociates from the receptor complex. Our data show that the kinetics of association of p90 and IRAK-1 with the receptor were similar, suggesting that the two may be identical. However, it could be that p90 is phosphorylated *in vivo* after IL-1 stimulation and remains associated with the receptor, leaving fewer sites available for its *in vitro* phosphorylation. In this case it could be a substrate for IRAK-4 or IRAK-1. In the preparative experiment where large amounts of cells were used (3×10^9 cells per immunoprecipitation), p90 appeared as a more diffuse band than in the analytical scale experiments where much smaller amounts of cells were used (10^8 or 0.5×10^8 cells per immunoprecipitation). This is mainly due to the fact that in the large scale experiments the amount of p90 that was immunoprecipitated with IL-1RI was much higher. A part of it co-migrated with very large amounts of IL-1RI and IL-1RAcP. IL-1RI and its accessory protein are very likely highly glycosylated, migrate as diffuse bands in the gel, and might possibly lead to p90 being diffuse also. In addition in this large scale experiment an 8–16% SDS-polyacrylamide gel was used (after trying a lot of different gradients) to better separate the p60 protein from the other proteins in the immunoprecipitates, whereas in the smaller scale experiments, 12.5% SDS-polyacrylamide gels were used. In all the analytical scale experiments where the number of cells used was smaller and thus the amounts of IL-1RI, IL-1RAcP, and IRAK-1 were lower, p90 appeared as a more discrete band when the immunoprecipitates were used for *in vitro* phosphorylation reactions, and IRAK-1 and IL-1RAcP also appeared as more discrete bands when the immunoprecipitates were used for immunoblotting.

Migration of the p36 protein on one-dimensional SDS-PAGE gels was retarded compared with glyceraldehyde-3-phosphate dehydrogenase and MyD88. Glyceraldehyde-3-phosphate dehydrogenase probably contaminated the immunoprecipitates as it is an abundant cytoplasmic protein

and was detected by MS even in receptor complexes from unstimulated cells. Phosphorylation of MyD88 in response to IL-1 stimulation has not been reported, although signaling via Toll-like receptor 4 results in its tyrosine phosphorylation (50). Thus, it is likely that MyD88 can also be phosphorylated in IL-1 signaling.

In conclusion, this is the only direct analysis of the assembly of the endogenous IL-1RI signaling complex. We demonstrated that the first component of the IL-1RI signaling complex ever to be observed to become phosphorylated *in vitro* (the protein p60) (39) is actually IRAK-4. Its *in vitro* phosphorylation occurred only on serines and threonines. Our work also provides a new mechanistic insight into the initiation of IL-1RI signaling: stable association of IL-1RAcP, MyD88, and IRAK-4 upon the binding of IL-1 to its receptor IL-1RI. Only IRAK-1 associates with the receptor transiently. To our knowledge, there is no other similar study of any other receptor complex from the IL-1/Toll-like receptor family analyzed at its endogenous levels. The Toll-like receptor signaling complexes and that of IL-1RI contain several common proteins such as MyD88, IRAK-1, and IRAK-4. Thus, our work, which overcame difficulties to keep the immunoprecipitated receptor complexes intact and adequately free of contaminating proteins so they can be successfully analyzed by MS (identifying for the first time several endogenous proteins at once), could be very useful in the application of a similar method to other members of the IL-1/Toll-like receptor family or to other receptors in general.

Acknowledgment—We thank John Sims for providing us with large amounts of M5 antibody, the EL4 6.1 cell line, and general support.

* This work was supported by the Arthritis Research Campaign, United Kingdom and by Science Foundation Ireland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: School of Biochemistry and Immunology, Trinity College, Dublin 2, Ireland. Tel.: 353-1-8962449; Fax: 353-1-6772400; E-mail: brikosc@tcd.ie.

‖ Both authors contributed equally to this work.

REFERENCES

1. Dinarello, C. A. (1996) Biologic basis for interleukin-1 in disease. *Blood* **87,** 2095–2147
2. Labriola-Tompkins, E., Chandran, C., Varnell, T. A., Madison, V. S., and Ju, G. (1993) Structure-function analysis of human IL-1α: identification of residues required for binding to the human type I IL-1 receptor. *Protein Eng.* **6,** 535–539
3. Grutter, M. G., van Oostrum, J., Priestle, J. P., Edelmann, E., Joss, U., Feige, U., Vosbeck, K., and Schmitz, A. (1994) A mutational analysis of receptor binding sites of interleukin-1β: differences in binding of human interleukin-1β muteins to human and mouse receptors. *Protein Eng.* **7,** 663–671
4. Dower, S. K., Kronheim, S. R., March, C. J., Conlon, P. J., Hopp, T. P., Gillis, S., and Urdal, D. L. (1985) Detection and characterization of high affinity plasma membrane receptors for human interleukin 1. *J. Exp. Med.* **162,** 501–515
5. Bird, T. A., Gearing, A. J., and Saklatvala, J. (1988) Murine interleukin 1 receptor. Direct identification by ligand blotting and purification to homogeneity of an interleukin 1-binding glycoprotein. *J. Biol. Chem.* **263,** 12063–12069
6. Urdal, D. L., Call, S. M., Jackson, J. L., and Dower, S. K. (1988) Affinity purification and chemical analysis of the interleukin-1 receptor. *J. Biol. Chem.* **263,** 2870–2877
7. Karin, M., and Ben-Neriah, Y. (2000) Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annu. Rev. Immunol.* **18,** 621–663
8. Chang, L., and Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature* **410,** 37–40
9. Greenfeder, S. A., Nunes, P., Kwee, L., Labow, M., Chizzonite, R. A., and Ju, G. (1995) Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. *J. Biol. Chem.* **270,** 13757–13765
10. Korherr, C., Hofmeister, R., Wesche, H., and Falk, W. (1997) A critical role for interleukin-1 receptor accessory protein in interleukin-1 signaling. *Eur. J. Immunol.* **27,** 262–267
11. Radons, J., Gabler, S., Wesche, H., Korherr, C., Hofmeister, R., and Falk, W. (2002) Identification of essential regions in the cytoplasmic tail of interleukin-1 receptor accessory protein critical for interleukin-1 signaling. *J. Biol. Chem.* **277,** 16456–16463
12. Wesche, H., Henzel, W. J., Shillinglaw, W., Li, S., and Cao, Z. (1997) MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* **7,** 837–847
13. Burns, K., Martinon, F., Esslinger, C., Pahl, H., Schneider, P., Bodmer, J. L., Di Marco, F., French, L., and Tschopp, J. (1998) MyD88, an adapter protein involved in interleukin-1 signaling. *J. Biol. Chem.* **273,** 12203–12209
14. Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C. A. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol. Cell* **2,** 253–258
15. Burns, K., Clatworthy, J., Martin, L., Martinon, F., Plumpton, C., Maschera, B., Lewis, A., Ray, K., Tschopp, J., and Volpe, F. (2000) Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. *Nat. Cell Biol.* **2,** 346–351
16. Cao, Z., Henzel, W. J., and Gao, X. (1996) IRAK: a kinase associated with the interleukin-1 receptor. *Science* **271,** 1128–1131
17. 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
18. 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
19. 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
20. Cao, Z., Xiong, J., Takeuchi, M., Kurama, T., and Goeddel, D. V. (1996) TRAF6 is a signal transducer for interleukin-1. *Nature* **383,** 443–446
21. 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 NFκB. *J. Biol. Chem.* **276,** 41661–41667
22. Jiang, Z., Ninomiya-Tsuji, J., Qian, Y., Matsumoto, K., and Li, X. (2002) Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. *Mol. Cell. Biol.* **22,** 7158–7167
23. Takaesu, G., Kishida, S., Hiyama, A., Yamaguchi, K., Shibuya, H., Irie, K., Ninomiya-Tsuji, J., and Matsumoto, K. (2000) TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. *Mol. Cell* **5,** 649–658
24. Holtmann, H., Enninga, J., Kalble, S., Thiefes, A., Dorrie, A., Broemer, M., Winzen, R., Wilhelm, A., Ninomiya-Tsuji, J., Matsumoto, K., Resch, K., and Kracht, M. (2001) The MAPK kinase kinase TAK1 plays a central role in coupling the interleukin-1 receptor to both transcriptional and RNA-targeted mechanisms of gene regulation. *J. Biol. Chem.* **276,** 3508–3516
25. Kishimoto, K., Matsumoto, K., and Ninomiya-Tsuji, J. (2000) TAK1 mitogen-activated protein kinase kinase kinase is activated by autophosphorylation within its activation loop. *J. Biol. Chem.* **275,** 7359–7364
26. Sakurai, H., Miyoshi, H., Mizukami, J., and Sugita, T. (2000) Phosphorylation-dependent activation of TAK1 mitogen-activated protein kinase kinase kinase by TAB1. *FEBS Lett.* **474,** 141–145
27. Takaesu, G., Surabhi, R. M., Park, K. J., Ninomiya-Tsuji, J., Matsumoto, K., and Gaynor, R. B. (2003) TAK1 is critical for IκB kinase-mediated activation of the NF-κB pathway. *J. Mol. Biol.* **326,** 105–115

28. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature* **388,** 548–554
29. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. *Science* **278,** 860–866
30. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation. *Cell* **91,** 243–252
31. Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E., and Hagiwara, M. (1996) A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J. Biol. Chem.* **271,** 13675–13679
32. Shirakabe, K., Yamaguchi, K., Shibuya, H., Irie, K., Matsuda, S., Moriguchi, T., Gotoh, Y., Matsumoto, K., and Nishida, E. (1997) TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. *J. Biol. Chem.* **272,** 8141–8144
33. Knop, J., Wesche, H., Lang, D., and Martin, M. U. (1998) Effects of over-expression of IL-1 receptor-associated kinase on NFκB activation, IL-2 production and stress-activated protein kinases in the murine T cell line EL4. *Eur. J. Immunol.* **28,** 3100–3109
34. Li, X., Commane, 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
35. 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
36. Maschera, B., Ray, K., Burns, K., and Volpe, F. (1999) Overexpression of an enzymically inactive interleukin-1-receptor-associated kinase activates nuclear factor-κB. *Biochem. J.* **339,** 227–231
37. Suzuki, N., Suzuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., Penninger, J. M., Wesche, H., Ohashi, P. S., Mak, T. W., and Yeh, W. C. (2002) Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature* **416,** 750–756
38. Picard, C., Puel, A., Bonnet, M., Ku, C. L., Bustamante, J., Yang, K., Soudais, C., Dupuis, S., Feinberg, J., Fieschi, C., Elbim, C., Hitchcock, R., Lammas, D., Davies, G., Al-Ghonaium, A., Al-Rayes, H., Al-Jumaah, S., Al-Hajjar, S., Al-Mohsen, I. Z., Frayha, H. H., Rucker, R., Hawn, T. R., Aderem, A., Tufenkeji, H., Haraguchi, S., Day, N. K., Good, R. A., Gougerot-Pocidalo, M. A., Ozinsky, A., and Casanova, J. L. (2003) Pyogenic bacterial infections in humans with IRAK-4 deficiency. *Science* **299,** 2076–2079
39. Martin, M., Bol, G. F., Eriksson, A., Resch, K., and Brigelius-Flohe, R. (1994) Interleukin-1-induced activation of a protein kinase co-precipitating with the type I interleukin-1 receptor in T cells. *Eur. J. Immunol.* **24,** 1566–1571
40. Gallis, B., Prickett, K. S., Jackson, J., Slack, J., Schooley, K., Sims, J. E., and Dower, S. K. (1989) IL-1 induces rapid phosphorylation of the IL-1 receptor. *J. Immunol.* **143,** 3235–3240
41. Blume-Jensen, P., and Hunter, T. (2001) Two-dimensional phosphoamino acid analysis. *Methods Mol. Biol.* **124,** 49–65
42. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68,** 850–858
43. Wait, R., Gianazza, E., Eberini, I., Sironi, L., Dunn, M. J., Gemeiner, M., and Miller, I. (2001) Proteins of rat serum, urine, and cerebrospinal fluid: VI. Further protein identifications and interstrain comparison. *Electrophoresis* **22,** 3043–3052
44. Wait, R., Miller, I., Eberini, I., Cairoli, F., Veronesi, C., Battocchio, M., Gemeiner, M., and Gianazza, E. (2002) Strategies for proteomics with incompletely characterized genomes: the proteome of Bos taurus serum. *Electrophoresis* **23,** 3418–3427
45. Bol, G., Kreuzer, O. J., and Brigelius-Flohe, R. (2000) Translocation of the interleukin-1 receptor-associated kinase-1 (IRAK-1) into the nucleus. *FEBS Lett.* **477,** 73–78
46. Yamin, T. 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
47. Jiang, Z., Johnson, H. J., Nie, H., Qin, J., Bird, T. A., and Li, X. (2003) Pellino 1 is required for interleukin-1 (IL-1)-mediated signaling through its interaction with the IL-1 receptor-associated kinase 4 (IRAK4)-IRAK-tumor necrosis factor receptor-associated factor 6 (TRAF6) complex. *J. Biol. Chem.* **278,** 10952–10956
48. Burns, K., Janssens, S., Brissoni, B., Olivos, N., Beyaert, R., and Tschopp, J. (2003) Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. *J. Exp. Med.* **197,** 263–268
49. Gay, N. J., Gangloff, M., and Weber, A. N. (2006) Toll-like receptors as molecular switches. *Nat. Rev. Immunol.* **6,** 693–698
50. Ojaniemi, M., Glumoff, V., Harju, K., Liljeroos, M., Vuori, K., and Hallman, M. (2003) Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. *Eur. J. Immunol.* **33,** 597–605