IRAK1b, a Novel Alternative Splice Variant of Interleukin-1 Receptor-associated Kinase (IRAK), Mediates Interleukin-1 Signaling and Has Prolonged Stability*

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Interleukin-1 (IL-1) is a pleiotropic cytokine essential for initiation of the immune response to infections and stress. IL-1 interacts with its type I receptor (IL-1RI) and triggers a number of intracellular signaling cascades leading to activation of transcription factors, transcriptional up-regulation of target genes, and mRNA stabilization. IL-1RI-associated kinase-1 (IRAK1) is a membrane proximal serine-threonine kinase involved in IL-1 signaling that becomes phosphorylated and progressively degraded in response to IL-1 induction. We have identified a novel variant of IRAK1, which we have named IRAK1b, that arises from the use of an alternative 5′-acceptor splice site defined by sequence within exon 12 of IRAK1. IRAK1b mRNA exhibits wide tissue expression and is evolutionarily conserved in both mouse and human. IRAK1b can activate the transcription factor nuclear factor-κB and interacts with the IL-1 signaling factors Toll-interacting protein and tumor necrosis factor receptor-associated factor 6. It forms homodimers and heterodimers with the previously described isoform of IRAK1. We show that the IRAK1b protein is kinase-inactive and that, unlike IRAK1, its levels remain constant after IL-1 induction. The presence of an alternative splice variant of IRAK1, which is functionally active and highly stable following IL-1 stimulation, adds further complexity to the control mechanisms that govern IL-1 signaling.

Infections, tissue injury, and/or stress trigger monocytes and macrophages to produce interleukin-1 (IL-1),1 the cytokine that orchestrates much of the systemic acute phase response, the net effect of which is to neutralize the underlying physiological challenge (reviewed in Ref. 1). Systemically, IL-1 stimulates fever, vasodilation, and muscle contractions; at the cellular level it induces cells to adopt an enhanced “host defense” phenotype by eliciting radical changes in their protein expression profiles by modulating mRNA processing and stability, protein translation, and gene transcription (1). The latter is achieved by activating transcription factors, e.g. nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). Signaling by IL-1 depends on its engagement of the transmembrane IL-1 receptor type I (IL-1RI) and IL-1RI accessory protein (IL-1RAcP) (Ref. 2 and references therein). Binding of IL-1 to the extracellular domains of IL-1RI and IL-1RAcP is followed by recruitment of the intracellular adapter protein myeloid differentiation factor (MyD88) (3–6) and a number of kinases to the evolving IL-1-1IL-1RI-IL-1RAcP complex. Depending on cell type, these kinases may include IL-1RI-associated kinase-1 (IRAK1), which is expressed in all tissues (7), IRAK2, which has a narrower cellular distribution (3), and IRAK-M, which is mainly restricted to cells of myeloid origin (8). Phosphatidylinositol 3-kinase has also been implicated as an early component of the IL-1 signaling cascade (9). Once established, the cascade progresses through the stepwise activation/recruitment of several additional intermediates, including tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), TRAF6 binding protein, transforming growth factor-β-activated kinase 1 (TAK1), two TAK1 binding proteins (TAB1 and TAB2), and NF-κB-inducing kinase (10–15). Finally, the inhibitor of NF-κB (IκB) is phosphorylated by the resulting IκB kinase complex and degraded. This allows NF-κB to translocate to the nucleus where it activates transcription of a wide range of genes that are important for immune function and inflammation (16). The branches of the IL-1 signaling pathway that leads to activation of AP-1 and mRNA stabilization may diverge from the NF-κB-activating branch of the cascade at TAK1/TAB1 (Ref. 17 and references therein). However, other studies suggest that the pathways diverge at IRAK1 or even earlier (18, 19).

IRAK1 is a protein of 714 amino acids that has two known functional domains: an N-terminal death domain, which is involved in protein-protein interactions with MyD88 and Toll-interacting protein (Tollip), and a centrally positioned Ser/Thr kinase domain (3, 5–7, 20). Several studies have shown that the kinase activity is not necessary for IRAK1 to be functional (21–23). In un-stimulated cells IRAK1 is associated with Tollip; however, following stimulation with IL-1, its recruitment to the IL-1-1IL-1RI-IL-1RAcP complex is facilitated by the interaction between Tollip and IL-1RAcP (24). IRAK1 becomes phosphorylated, dissociates from Tollip, and is degraded by proteasomes (24, 25). Studies to date indicate that the phosphorylation of IRAK1 is mediated by IRAK1 itself (7, 20), although it remains a possibility that additional kinases are also involved.
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(23). It has furthermore been suggested, but not yet established, that IL-1-mediated degradation of IRAK1 may be the mechanism whereby cells become desensitized after prolonged exposure to IL-1 (25). Because IRAK1 is also involved in the signaling pathway from Toll receptors induced by lipopolysaccharide (see Ref. 26 for additional references), it has also been suggested that a similar mechanism plays a role in the desensitization of monocytes and neutrophils to bacterial lipopolysaccharide, which is a feature of sepsis (26).

Alternative splicing is one of the mechanisms whereby an increased complexity in the number of functionally distinct protein products may be generated from a fixed pool of genes in the genome (27). We have identified a novel alternatively spliced variant of IRAK1 mRNA. It encodes an IRAK1 protein that has functional activity similar to that of the previously described IRAK1 isoform but is biologically distinct in that it is relatively much more stable after IL-1 activation. The existence of alternative forms of IRAK1 with very different capacities to support IL-1 signaling over time has important implications for the initiation and maintenance of inflammatory processes.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cultures**—Human hepatoma cells (HepG2) and human embryonic kidney cells (293) were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium with 25 mM HEPES and glutamax-I (t-alanyl-l-glutamine) supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 0.01 mM nonessential amino acids, and 50 μg/ml gentamicin (Life Technologies, Inc., Grand Island, NY). For protein harvesting 106 cells were used per data point. For transfection experiments 2.5 × 105 cells were used per data point. Treatments were performed in duplicate or triplicate. All experiments were repeated at least twice. For transfection experiments, cells were grown to ∼50% confluence and transfected with 2–3 μg of total DNA using FuGene6 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. In experiments where cells were transfected with varying amounts of expression vectors, the total DNA used in each transfection was held constant by co-transfecting appropriate amounts of empty vector. Cells were treated with medium (control) or IL-1β (10 ng/ml, National Cancer Institute, Frederick, MD) for 20 h. For indirect immunofluorescence reporter assays 24 h after transfection, cells were rinsed twice with PBS prior to the addition of lysis buffer. Beads were resuspended in SDS-PAGE sample buffer, and proteins were separated by SDS-PAGE with 4–12% Bis-Tris gels (NuPAGE, Invitrogen).

**Western Blotting and Immunoprecipitation**—Total proteins were extracted on ice in 50 mM HEPES, 150 mM NaCl, 20 mM β-glycerophosphate, 5 mM Na著名的EDTA, 1 mM Na3VO4, 5 mM para-nitrophenyl phosphate, 2 mM diethiothreitol, 10% (v/v) Protease Inhibitor Mixture (Sigma, St. Louis, MO), 1% Nonidet P-40. Cellular debris was removed by centrifugation at 10,000 × g for 10 min. Total protein extracts were separated in 6% SDS-polyacrylamide gel electrophoresis (PAGE) gels and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Excess protein binding sites were blocked with 5% nonfat milk in PBS. Immunodetection of IRAK1 was performed using polyclonal rabbit anti-IRAK1 (IRAK-1 (H273), Santa Cruz Biotechnology, Santa Cruz, CA), horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Santa Cruz Biotechnology), and enhanced chemiluminescence (ECL Western blotting detection reagents, Amersham Pharmacia Biotech). His-tagged polyclonal rabbit antibody (anti-his epitope) was used in Western blotting experiments (Santa Cruz Biotechnology). FLAG-tagged proteins were immunoprecipitated with ANTI-FLAG M2 (Sigma) monoclonal antibodies and agarose-conjugated protein A (Life Technologies, Inc.) at 4 °C for 4 h.

**Kinase Assays**—Washed immunoprecipitates were resuspended in kinase buffer (20 mM Tris, pH 7.6, 1 mM dithiothreitol, 20 mM MgCl2, 20 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 20 mM para-nitrophenylphosphate, 10% (v/v) Protease Inhibitor Mixture (Sigma, St. Louis, MO), 1% Nonidet P-40, 1 mM ATP). To each sample 2 μg of mouse basic protein (MBP, Sigma) was added. Each sample was divided into two aliquots, to one of which 10 μg of [γ-32P]ATP (Amersham Pharmacia Biotech) was added. Samples were incubated at 37 °C for 30 min. Reactions were stopped by addition of SDS-PAGE sample buffer and boiling for 3 min. Proteins were separated in 4–12% Bis-Tris SDS-PAGE gels. Proteins were detected by autoradiography.

**Computer Analyses**—Computer analyses were performed through the Baylor College of Medicine search launcher available at searchlauncher.bcm.tmc.edu. The programs HSPL (prediction of splice sites in human DNA sequences) and FEXH (prediction of internal, 5’, and 3’-exons in human DNA sequences) were used to predict splice sites (30) in the IRAK1 sequence.

**RESULTS AND DISCUSSION**

**Identification of Potential Alternatively Spliced cDNA Clones**—IRAK1 is an essential component of the IL-1 signaling cascade. It has been suggested that degradation of IRAK1 is mediated by caspase-1, which cleaves the IRAK1 kinase domain and liberates IRAK1 from association with Toll and lipopolysaccharide after prolonged exposure to these agents (25, 26). To test this hypothesis we generated a range of IRAK1 expression vectors. Primers for amplification of IRAK1 sequences were designed from GenBank access number L76191, and the entire coding sequence was amplified by PCR using proofreading DNA polymerases. The re-
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Expression of Alternately Spliced Variants of Human IRAK1—IRAK1α has previously been shown to be expressed in a wide range of cell types as an ~3.5-kb mRNA (7, 20). The similarity in size between the IRAK1α and IRAK1β mRNAs precludes their resolution by Northern blot analysis. We therefore chose a RT-PCR approach to identify cell types that express IRAK1α mRNA. Using this method, we were able to detect IRAK1α mRNA expression in a wide range of tissues. The ratio of IRAK1α to IRAK1β mRNA was similar in all tissues tested. The ratio of IRAK1α mRNA to IRAK1β mRNA was similar in all samples. We next wished to establish whether the putative IRAK1β protein could be detected in protein extracts from HepG2 cells. Cells were transfected with pCINeo expression plasmids encoding either IRAK1α or IRAK1β. The protein levels of IRAK1α and IRAK1β were measured using Western blots of total protein extracts. A 250-fold excess of cell extracts from untransfected cells was used relative to extracts from transfected cells. Two bands with mobilities identical to those observed in cells transfected with pCINeo were observed in extracts from untransfected cells (Fig. 2A). We expected the size of the IRAK1β cDNA to be ~351 bp, and we concluded that IRAK1β mRNA is co-expressed with IRAK1α mRNA in all tissues tested. The ratio of IRAK1α mRNA to IRAK1β mRNA was similar in all samples.

Evolutionary Conservation of IRAK1β—The mouse IRAK1α mRNA sequence (IRAK1/mPLK, GenBank® accession number Y52112) shows a high degree of similarity to the human IRAK1α sequence. The nucleotide sequence, which is not present in IRAK1β mRNA, is underlined. The schematic representation of IRAK1α polypeptide. The amino acid sequence, which is not present in IRAK1β protein, is shown above the diagram of the IRAK1α domain organization and its relative location is indicated. The positions of the two serine residues (\*), and the single lysine and tyrosine residues (\#) within this sequence are indicated. The tripptide that is absent from mouse IRAK1a is underlined.}

Fig. 1. Exon 12 of IRAK1 and position of alternative splice sites. A, exon 12 of the human IRAK1 gene (GenBank® accession number Y52112) is shown in uppercase letters and segments of surrounding intron sequence are shown in lowercase letters. The nucleotide sequence, which is not present in IRAK1β mRNA, is underlined. B, schematic representation of IRAK1α polypeptide. The amino acid sequence, which is not present in IRAK1β protein, is shown above the diagram of the IRAK1α domain organization and its relative location is indicated. The positions of the two serine residues (\*), and the single lysine and tyrosine residues (\#) within this sequence are indicated. The tripptide that is absent from mouse IRAK1a is underlined.

Fig. 2. Expression of alternatively spliced variants of IRAK1. A, detection of IRAK1α mRNA splice variants in human tissues. IRAK1α cDNAs were amplified as described under “Experimental Procedures.” Positions of the IRAK1α (441 bp) and IRAK1β (351 bp) alternatively spliced cDNAs products are indicated. B, detection of IRAK1α proteins derived from alternatively spliced IRAK1α and IRAK1β mRNAs. Human HeLa cells were transfected with a kinase-inactive IRAK1α (lane labeled kiIRAK1α) or IRAK1β (lane labeled IRAK1β) expression construct or left untransfected (lane labeled HepG2). IRAK1α proteins were detected using Western blots of total protein extracts. A 250-fold excess of cell extract from untransfected cells was used relative to extracts from transfected cells. Two bands with mobilities identical to those observed in cells transfected with pCINeo were observed in extracts from untransfected cells (Fig. 2B). We expected the size of the IRAK1β cDNA to be ~351 bp, and we concluded that IRAK1β mRNA is co-expressed with IRAK1α mRNA in all tissues tested. The ratio of IRAK1α mRNA to IRAK1β mRNA was similar in all samples.
NF-κB expression constructs (see “Experimental Procedures”). The degree of transfection efficiency. The proportional difference in NF-κB activation between IRAK1a and IRAK1b is similar to that previously reported between IRAK1a and either IRAK2 or IRAK-M (8), i.e. IRAK1b appears to be as efficient as IRAK2 and IRAK-M at activating NF-κB.

**Protein-Protein Interaction with IRAK1 Splice Variants—** The lower efficiency of NF-κB activation by IRAK1b relative to that effected by IRAK1a prompted us to investigate if there are measurable differences in the interactions of IRAK1a and IRAK1b with the downstream signaling component TRAF6. His-tagged IRAK1 proteins were expressed either alone or with His-tagged TRAF6 in 293 cells. TRAF6 was immunoprecipitated from cellular extracts with a TRAF6-specific antibody, and co-immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting. All three IRAK1 proteins, IRAK1b, kiIRAK1a, and IRAK1a co-precipitated equally well with TRAF6 (Fig. 4A). Neither of the IRAK1 proteins could be immunoprecipitated with anti-TRAF6 in the absence of co-transfected TRAF6 (not shown) verifying that direct interaction with TRAF6 is necessary for co-immunoprecipitation. Furthermore, His-tagged Tollip co-expressed with TRAF6 could not be co-immunoprecipitated with TRAF6, confirming that the IRAK1-TRAF6 interaction is specific (Fig. 4A).

It has been shown that IRAK1a forms homodimers and heterodimers with IRAK2 and IRAK-M (8). We therefore next examined if IRAK1b would form homo- and heterodimers with itself and IRAK1a, respectively. Cells were transfected with expression vectors for the FLAG-tagged IRAK1 proteins IRAK1b, kiIRAK1a, or IRAK1a or the FLAG-tagged BAP protein (a control that is not involved in IL-1 signaling). In addition all cells were transfected with an expression vector encoding His-tagged IRAK1b. FLAG-tagged proteins were immunoprecipitated using anti-FLAG antibodies. Immunoprecipitated and co-immunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting. His-tagged IRAK1b was detected using anti-His antibodies and was found to co-immunoprecipitate equally well with all the FLAG-tagged IRAK1 proteins, i.e. IRAK1b, kiIRAK1a, and IRAK1a (Fig. 4B), establishing that IRAK1b can form both homodimers and heterodimers with IRAK1a. His-tagged IRAK1b did not co-immunoprecipitate with FLAG-tagged BAP (Fig. 4B), demonstrating that the generation of all of the above classes of dimers is specific. FLAG-tagged immunoprecipitated proteins were detected with anti-FLAG to verify efficient precipitation (Fig. 4B).

To investigate if IRAK1b interacts with signaling components upstream of IRAK1 in the IL-1 signaling cascade, we expressed His-tagged Tollip with FLAG-BAP, FLAG-IRAK1b, FLAG-kiIRAK1a, or FLAG-IRAK1a. FLAG-tagged proteins were immunoprecipitated, and proteins were analyzed as described above. His-Tollip co-immunoprecipitated only with FLAG-tagged IRAK1b and kiIRAK1a and not with FLAG-tagged BAP or IRAK1a. The latter is consistent with the previous observation by Burns et al. (24) that IRAK1a dissociates from Tollip when phosphorylated.

Tollip is the factor that is associated with IRAK1 before IL-1 treatment and is responsible for recruiting IRAK1 to the IL-1/IL-1RI/IL-1RaCp complex. The ability of Tollip to specifically co-immunoprecipitate with IRAK1b suggests that IRAK1b is recruited to the IL-1/IL-1RI/IL-1RaCp complex in response to IL-1 stimulation and provides further support for our conclusion that IRAK1b is involved in IL-1 signaling.

**Phosphorylation and Degradation of IRAK1 Splice Variants—**

U56773) was aligned with that of the human IRAK1 mRNA. The two splice sites for IRAK1a and IRAK1b are perfectly conserved in the mouse sequence (not shown). Intriguingly, three amino acid residues, which are present in the region of the human IRAK1 that is spliced out of human IRAK1b, are absent from the mouse gene (Fig. 1B). A proportional quantitative RT-PCR method similar to the one described above for human IRAK1a and IRAK1b mRNA splice variants was developed for the mouse putative IRAK1 mRNA splice variants. Both cDNA products could be amplified from total RNA extracted from mouse liver, kidney, and testis (Fig. 2C). The retention of the IRAK1b alternative splice variant in two evolutionarily distant mammalian species suggests that it has an important physiological function.

**PELLE,** the Drosophila homologue of IRAK1, contains death and kinase domains that are similar to those present in IRAK1; however, the PELLE peptide sequence ends immediately after the kinase domain and the full-length protein is only 501 amino acid residues long (31). PELLE therefore does not contain the C-terminal region, which is subject to alternative splicing in mammalian IRAK1. The presence of this domain in both human IRAK1 mRNA splice variants is conserved in the mouse sequence (not shown). Intriguingly, the two splice sites for IRAK1a and IRAK1b are perfectly conserved in the mouse sequence (not shown). In this context it is intriguing that the C-terminal domain incorporates potential undefined, that are specific to mammals. In this context it is intriguing that the C-terminal domain incorporates potential undefined, that are specific to mammals.
ants—IRAK1a undergoes autophosphorylation in response to IL-1, and when overexpressed in the absence of cytokine (5, 7, 8, 20, 25). Phosphorylated IRAK1a exhibits slower mobility in PAGE. It also has been shown that phosphorylated IRAK1a is degraded by the ubiquitin-proteasome pathway. We observed that, when overexpressed, IRAK1a migrates with an apparent molecular mass of 110–120 kDa (Fig. 5A), whereas both the endogenous unphosphorylated IRAK1a and the transfected kiIRAK1a mutant migrate at 76 kDa, as would be expected of the unmodified polypeptide. Unlike IRAK1a, IRAK1b migrates at the expected 73-kDa position when overexpressed in HepG2 cells (Fig. 5A), indicating that IRAK1b does not undergo autophosphorylation.

To investigate the consequences of IL-1 induction on the endogenous IRAK1 isoforms, HepG2 cells were treated with IL-1 for various lengths of time prior to Western blot analysis of IRAK1 proteins. As previously described in 293 and MRC-5 cells (7, 25) the 76-kDa IRAK1a band gradually disappeared as bands of slower mobility appeared (Fig. 5B). At least four phosphorylated forms of IRAK1a with apparent molecular masses of 79, 83, 90, and 110–120 kDa appear. No changes in intensity of the 73-kDa band were observed (Fig. 5B), suggesting that, unlike IRAK1a, IRAK1b does not become phosphorylated and degraded in response to IL-1. In unstimulated cells, and at the earliest time points after cytokine induction, IRAK1a is the dominant IRAK1 isoform; however, at later time points post-stimulus the levels of the two unphosphorylated isoforms are almost equimolar. After prolonged exposure (24 h) of cells to IL-1, levels of IRAK1b were as high as, and in some experiments higher than, those of IRAK1a (Fig. 5C).

**Half-lives of IRAK1 Proteins**—It has been reported that in MRC-5 cells the 76-kDa non-phosphorylated IRAK1a band has a $t_{1/2}$ of only about 2 min in response to IL-1 due to phosphorylation and degradation (25). We used ImageQuaNT technology applied to Western blots (Fig. 5B) to quantify the amount of non-phosphorylated 76-kDa IRAK1a protein remaining after IL-1 treatment. The turnover of non-phosphorylated IRAK1a appears to be biphasic. Immediately following IL-1 treatment it has a $t_{1/2}$ of 15 min (Fig. 6A). Shortly thereafter there is an apparent transition to a $t_{1/2}$ value of at least 2 h (Fig. 6A). The longer half-life of IRAK1a in the period immediately after IL-1 treatment in HepG2 compared with that observed in MRC-5 cells may reflect tissue-specific differences in the rate of signal factor recruitment and/or differences in levels of catalytic enzymes.

To further investigate the half-lives of IRAK1a and IRAK1b, we performed pulse-chase experiments. FLAG-IRAK1a, FLAG-kiIRAK1a, or FLAG-IRAK1b were expressed in HepG2 cells and labeled with $^{35}$S-methionine. Cells were treated with medium only or IL-1 and the $^{35}$S-labeled pro-
Cells were treated with medium only (triangles) or IL-1 (circles). Hyperphosphorylated FLAG-tagged IRAK1a (PP-IRAK1a) was expressed in HepG2 cells for pulse-chase experiments as outlined above. Cells were treated with IL-1, and amounts of endogenous non-phosphorylated IRAK1a at later stages of hyperphosphorylated IRAK1a when overexpressed in the absence of IL-1 (Fig. 5A) and the much lower level of accumulation of the endogenous hyperphosphorylated IRAK1a at later time points following IL-1 treatment (Fig. 5B). To determine the half-life of the non-phosphorylated IRAK1a in the absence of IL-1, we used the kinase-inactive mutant kiIRAK1a as a surrogate to allow us to overexpress FLAG-tagged protein without autophosphorylation (Fig. 5A). In pulse-chase experiments kiIRAK1a has a $t_{1/2}$ of $\sim$7 h in the absence of IL-1 (kiIRAK1a/Med, Fig. 6B) and an essentially similar $t_{1/2}$ of 6 h in the presence of IL-1 (kiIRAK1a/IL1, Fig. 6B).

In parallel experiments IRAK1b has similar half-lives of 7 and 6 h in the absence and presence of IL-1, respectively (Fig. 6C). These results establish that, although in the absence of IL-1 IRAK1a and IRAK1b have almost identical half-lives, the turnover rate of IRAK1b is essentially unchanged in response to IL-1 whereas IRAK1a becomes dramatically destabilized due to phosphorylation.

It is possible that the differential stability of the two IRAK1 isoforms in response to IL-1 stimulation reflects fundamental differences in “functional kinetics.” Because IRAK1a is the dominant isoform in unstimulated cells it is likely to be the isoform that is predominantly recruited to the IL-1-IL-1R-1Rα/IKα complex during the initial stages of assembly. The strong activity and relative abundance of IRAK1a would result in the rapid attainment of the high levels of NF-κB mobilization that are desirable early in the acute phase response. However, to prevent chronic “overstimulation” of NF-κB-mediated gene expression in the medium to long term, it would be necessary to efficiently degrade IRAK1a. At such later stages of the acute phase response the replacement of IRAK1a with IRAK1b would facilitate a continued but less vigorous input into the IL-1 signaling cascade thereby allowing NF-κB activation and other downstream components to be maintained at a more modest and sustainable level. At the terminal stages of the acute phase response, additional control mechanisms may be implemented to ensure that the IL-1 signaling pathway is finally shut down.

An alternative or additional possibility is that IRAK1a and IRAK1b play different roles in supporting the various downstream branches of the IL-1 signaling cascade, e.g. activation of NF-κB, activation of AP-1, and mRNA stabilization. Their different half-lives following IL-1 activation may reflect different requirements for initiating and maintaining subpathways with particular subsets of target genes for specific amounts of time under a range of static and dynamically changing induction conditions.

Phosphorylation of IRAK1a Mutants—Two potential serine phosphorylation sites (Ser-536 and Ser-541), one potential tyrosine phosphorylation site (Tyr-515), and one potential ubiquitination site (Lys-520) are located within the peptide sequence that is present only in IRAK1a (Fig. 1B). To determine whether these sites are involved in hyperphosphorylation and hence destabilization of IRAK1a, we generated mutants that were deficient in one or more of these sites. To minimize steric changes serine residues were replaced with alanines, tyrosine with phenylalanine, and lysine with alanine. The IRAK1a mutants were overexpressed in cells following transient transfection, and protein extracts were analyzed by Western blotting. All mutant proteins (i.e. IRAK1a(S536A), IRAK1a(S541A), IRAK1a(S536A,S541A), IRAK1a(K520A), and IRAK1a(K520A,S536A,S541A)) migrated in SDS-PAGE at the same 110- to 120-kDa position as wild type-hyperphosphorylated (and possibly ubiquitinated) IRAK1a, indicating that the mutant proteins are most likely post-translationally modified to the same extent as IRAK1a (Fig. 7).

Kinase Activity of IRAK1 Splice Variants—The first 11 amino acid residues of the 30 residues that are spliced out from IRAK1b comprise the C-terminal end of the IRAK1a isoforms. Non-transfected HepG2 cells were treated with IL-1, and proteins were harvested at indicated time points. The amount of endogenous IRAK1a was quantified using Western blotting and ImageQuant technology. For pulse-chase experiments cells were transfected with appropriate expression vectors encoding Flag-tagged IRAK1 proteins. Proteins were labeled with $[^35]$S-methionine for 1 h (pulse). Medium was replaced with fresh medium without $[^35]$S-methionine (time point zero) with or without IL-1. Proteins were harvested at appropriate time points (chase). Flag-tagged IRAK1 proteins were immunoprecipitated using anti-FLAG antibodies and quantified using ImageQuant technology following SDS-PAGE. Levels of IRAK1 protein remaining after medium only (Med) or IL-1 (IL1) treatment are expressed as percentage of amount present at time point zero (defined as 100%) on an exponential scale. A, non-transfected cells were treated with IL-1, and amounts of endogenous non-phosphorylated IRAK1a were determined as described above (boxes). Hyperphosphorylated FLAG-tagged IRAK1a (PP-IRAK1a) was expressed in HepG2 cells for pulse-chase experiments as outlined above. Cells were treated with medium only (triangles) or IL-1 (circles). B, FLG-tagged kiIRAK1a (kiIRAK1a) was expressed in HepG2 cells for pulse-chase experiments as outlined above. Cells were treated with medium only (boxes) or IL-1 (triangles). C, FLAG-tagged IRAK1b (IRAK1b) was expressed in HepG2 cells for pulse-chase experiments as outlined above. Cells were treated with medium only (boxes) or IL-1 (triangles).
with ATP. The immunoprecipitated IRAK1 proteins served as templates for kinase reactions with myelin basic protein (MBP) as the exogenous target (lower panel). Kinase assay products were separated by SDS-PAGE and subjected to autoradiography.

kinase domain (Fig. 1B). This led us to speculate that the activity of the truncated kinase domain (306 remaining amino acid residues) in IRAK1b may be substantially different from that of IRAK1a, thereby explaining the lack of autophosphorylating capacity in IRAK1b and the consequent differential stabilities of IRAK1a and IRAK1b following IL-1 induction. To investigate this further, we performed in vitro kinase assays. FLAG-tagged IRAK1a, kiIRAK1a, and IRAK1b were overexpressed in cells and immunoprecipitated with anti-IRAK or anti-FLAG antibodies (use of either antibody gave identical results). Washed immunoprecipitates were then used in kinase reactions with myelin basic protein (MBP) as an exogenous target. Although an excess of the kiIRAK1a and IRAK1b was used, only IRAK1a was labeled with 32P (Fig. 5). This result suggests that IRAK1b is kinaseinactive. A trivial explanation may be that the shorter polypeptide sequence of IRAK1b mandates a structural change that prevents IRAK1b from autophosphorylating/becoming phosphorylated at the site (or sites) that becomes phosphorylated in IRAK1a. However, only in reactions containing IRAK1a were there higher levels of 32P incorporation into MBP (Fig. 8) than those seen in reactions from mock transfected cells (background), leading us to conclude that this is not the case and that IRAK1b is truly kinase-inactive.

The phosphorylation of IRAK1a is believed to be mediated by IRAK1a itself, but it is not clear whether one IRAK1a molecule phosphorylates another IRAK1a molecule or if IRAK1a undergoes autophosphorylation. Our observations that IRAK1a becomes phosphorylated in response to IL-1 whereas IRAK1b does not, in conjunction with the results of the co-immunoprecipitation experiments showing that IRAK1b can form dimers with IRAK1a, suggest that in vivo IRAK1a undergoes a genuine autophosphorylation and not an IRAK1a to IRAK1 transphosphorylation process.

**Conclusion**—We have described a novel alternatively spliced variant of IRAK1, which we have named IRAK1b. The splice variant is evolutionarily conserved in mammals and can activate NF-kB, suggesting that it plays an important role in IL-1 signaling. IRAK1b distinguishes itself biologically from the previously described isoform of IRAK1, IRAK1a, by not being capable of autophosphorylation and by being resistant to IL-1-mediated degradation. This leads us to propose a model of membrane proximal IL-1 signaling in which (i) IRAK1a is the isoform that is initially recruited to the IL-1-IL-1RI-IL-1RacP complex to rapidly initiate the intracellular IL-1 signaling cascade, and (ii) IRAK1a levels are subsequently dramatically reduced to blunt the acute phase response and to permit IRAK1b to have a more prominent role in mediating a slower but more sustained engagement of downstream components of the signaling cascade. The net effect in this model would be an orderly switch in the predominant IRAK1 isoform, which is recruited into the IL-1-IL-1RI-IL-RacP complex from IRAK1a to IRAK1b, that is accompanied by a transition in the kinetics of the downstream signaling events such that an early rapid vigorous cellular response to IL-1 is superceded by a sustained response characterized by a more stable modified phenotype.

Further studies of the role of IRAK1 in IL-1-dependent signal transduction in the context of the two alternative kinases IRAK2 and IRAK-M and other membrane proximal factors will further enhance our overall understanding of the means whereby cytokines elicit changes in cellular phenotype.

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