The interleukin-1 receptor-associated kinase (IRAK) was first described as a signal transducer for interleukin-1 (IL-1) and has later been implicated in signal transduction of other members of the Toll/IL-1 receptor family. We now report the identification and characterization of a novel IRAK-like molecule. In contrast to the ubiquitously expressed IRAK and IRAK-2, this new IRAK-like molecule is found mainly in cells of monomyeloid origin and is, therefore, designated IRAK-M. Although IRAK-M and IRAK-2 exhibit only a negligible autophosphorylation activity, they can reconstitute the IL-1 response in a 293 mutant cell line lacking IRAK. In addition, we show for the first time that members of the IRAK family are indispensable elements of lipopolysaccharide signal transduction. The discovery of IRAK-M adds another level of complexity to our understanding of signaling by members of the Toll/IL-1 receptor family.

The Toll/IL-1R receptor family consists of a large number of transmembrane proteins with conserved intracellular domains. Structural distinctions in the extracellular domains of these proteins divide this superfamily into two subgroups: the Toll-like receptors (TLRs) with leucine-rich repeats and IL-1R-related proteins with immunoglobulin-like motifs (1; for review, see Refs. 2 and 3). All members of the Toll/IL-1R family with known functions are involved in host defense. For example, the proinflammatory and immune regulatory cytokines IL-1 and IL-18 signal through members of the IL-1R-related protein subfamily (4, 5; for review, see Refs. 6 and 7), whereas TLR-2 and TLR-4 have been shown recently to mediate cellular response to bacterial lipopolysaccharide (LPS) (8–12).

The signal transduction pathways initiated by Toll/IL-1R family members ultimately lead to the activation of members of the rel and AP-1 family of transcription factors (for review, see Refs. 2, 3, 7, and 13). The receptor proximal signaling events of the proinflammatory cytokine IL-1 have been studied in detail. The first signaling event for IL-1 is the ligand-induced complex formation of IL-1RI and IL-1RAcP (14–17). The adaptor protein MyD88 is next recruited to this complex (18–20), which in turn enables the association of the IL-1-associated kinase (IRAK) (21–23). IRAK gets highly phosphorylated (21, 24), leaves the receptor complex, and interacts with TRAF6 (25). The IRAK-TRAF6 interaction triggers kinase cascades that lead to the activation of IxB kinases and c-Jun NH2-terminal kinase, which phosphorylate IxB and c-Jun, respectively (for review, see Refs. 7, 26, and 27).

Accumulating evidence indicates that several components of the IL-1 pathway are also utilized by other members of the Toll/IL-1R family. TLR-4 has been shown to interact with MyD88, IRAK, and TRAF6 upon overexpression (28, 29). Dominant negative mutants of MyD88 and TRAF6 can block LPS-induced NF-κB activation mediated by TLR-2 (9) as well as NF-κB activation induced by TLR-4 overexpression (28, 29). In addition, IL-18 induces IRAK phosphorylation and interaction with TRAF6 (30, 31). Further evidence implicating IL-1 signaling components in the IL-18 pathway was provided by the observation that targeted disruption of the myd88 gene in mice results in a loss of response to both IL-1 and IL-18 (32).

The role of IRAK in cytokine signaling is also supported by genetic studies. Embryonic fibroblasts derived from IRAK-deficient mice show reduced NF-κB and c-Jun NH2-terminal kinase activities after IL-1 treatment (33). IRAK knock-out mice are also severely compromised in IL-1-induced neutrophilia and IL-1-induced increase in serum levels of TNF and IL-6 (34). Furthermore, splenocytes derived from the IRAK knock-out mice produced significantly reduced amounts of interferon-γ in response to IL-18 (34). Nonetheless, in contrast to the phenotype of the MyD88 knock-out mice, IRAK-deficient mice retain partial IL-1 and IL-18 responses in all of the above mentioned assays, indicating the presence of a compensatory mechanism in animals.

An IRAK-related molecule designated IRAK-2 has been identified in a computer-assisted EST database search (19). IRAK-2 is able to interact with other IL-1 signal transducers and activate NF-κB upon overexpression in 293 cells. Here we report the identification and characterization of a novel IRAK-like molecule that has both similarities and differences compared with other members of the IRAK family. Utilizing an IRAK-deficient cell line, we present evidence that both IRAK-2 and the newly identified IRAK-like molecule can provide functional redundancy in signal transduction by the Toll/IL-1R receptor family.
with either 50 nM TPA/ionomycin (Sigma) or 10 μg/ml LPS (Sigma) for 48 h.

Cloning and Expression Vectors—IRAK-M cDNA was obtained from a λ phage library made of cDNA derived from human phytohemagglutinin-L-activated peripheral blood leukocytes in a low stringency hybridization using the EST clone AA840598 as a probe by following standard procedures (35). Mammalian expression vectors encoding NH2-terminal Flag-tagged IL-1RI, TRAF6, IRAK, IRAK K239S, and hemagglutinin-tagged N-IRAK (amino acids 1–215) as well as COOH-terminal Flag-tagged MyD88 have been described elsewhere (20). The NF-κB-dependent E-selectin-luciferase reporter plasmid pELAM-luc and pRSV-β-gal have also been described (36, 37). Constructs encoding COOH-terminal AU1-tagged IRAK-2, IRAK-2 K237A/K238A, and N-IRAK-2 (amino acids 1–95) were a kind gift of Vishva Dixit (19). Expression vectors for NH2-terminal Flag- or Myc-tagged IRAK-2, IRAK-M, and N-IRAK-M (amino acids 1–161) were constructed by FIG. 1.

Sequence alignment of IRAK/Pelle family members. Protein sequences of human IRAK-M, IRAK (accession number L76191), IRAK-2 (accession number AF026273), and Drosophila melanogaster Pelle (accession number L08476) were aligned using the ClustalW program. Identical and similar residues are indicated by dark and light shading, respectively. The GenBank accession number for human IRAK-M is AF113136.
inserting polymerase chain reaction-generated cDNA fragments in the mammalian expression pRK7 (38). IRAK-M K192A was constructed using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA).

RNA Preparation and Northern Blotting—Total RNA was isolated from various cell lines by guanidinium/phenol extraction (RNazol, Life Technologies) following the manufacturer’s instructions. 20 μg of RNA/ lane was separated on a 1.2% formaldehyde agarose gel and blotted to a nitrocellulose membrane (35). The blot was stained with 0.04% methylene blue to monitor sample loading. Hybridization and prehybridization were carried out at 65 °C in ExpressHyb solution (CLONTECH) following manufacturer-recommended procedures. Probes were labeled with [α-32P]dCTP using the random primer labeling kit (Amersham Pharmacia Biotech). Multiple tissue Northern blots were purchased from CLONTECH.

Reporter Assays—3 × 106 293L-1R or 293 IIa cells were seeded into six-well (35-mm) plates. Cells were transfected on the following day by the calcium phosphate precipitation method with 0.1 μg of pELAM-luc and 1 μg of pBSV-β-gal and the indicated amounts of expression constructs. After 24 h, the cells were stimulated with IL-1β (20 ng/ml), TNF-α (100 ng/ml), or LPS (E. coli serotype O111:B4, Sigma, 10 μg/ml) for 6–8 h before harvest. Luciferase activity and β-galactosidase activity were determined with the luciferase assay system (Promega) and chemoluminescence reagents from Tropix, respectively.

Immunoprecipitations and Immunoblotting—For coprecipitation of transfected proteins, 3 × 106 293 or 293 IIa cells were plated on 10-cm dishes and transfected on the following day with the indicated amounts of expression constructs. The total amount of DNA was kept constant in each transfection by adding empty vector. After 24 h, cells were collected, washed once with phosphate-buffered saline and lysed for 20 min on ice in 1 ml of lysis buffer containing 50 mM Hepes, pH 7.9, 250 mM NaCl, 20 mM MgCl2, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM p-nitrophenyl phosphate, 2 mM dithiothreitol, protease inhibitors (complete, Roche Molecular Biochemicals), and 1% Nonidet P-40. Cellular debris was removed by centrifugation twice at 15,000 g for 15 min. The cell lysate was incubated with 25 μl of M2 beads (Sigma) for 2 h at 4 °C with gentle rocking. After extensive washing with lysis buffer, proteins bound to the beads were either subjected to in vitro kinase assays (see below) or directly solubilized by boiling in SDS sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and blotted with the indicated antibodies. The reactive bands were visualized with horseradish peroxidase coupled to the appropriate secondary antibodies and the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech). For coprecipitation of transfected proteins, 3 × 106 293 or 293 IIa cells were plated on 10-cm dishes and transfected on the following day with the indicated amounts of expression constructs. The total amount of DNA was kept constant in each transfection by adding empty vector. After 24 h, cells were collected, washed once with phosphate-buffered saline and lysed for 20 min on ice in 1 ml of lysis buffer containing 50 mM Hepes, pH 7.9, 250 mM NaCl, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM p-nitrophenyl phosphate, 2 mM dithiothreitol, protease inhibitors (complete, Roche Molecular Biochemicals), and 1% Nonidet P-40. Cellular debris was removed by centrifugation twice at 15,000 g for 15 min. The cell lysate was incubated with 25 μl of M2 beads (Sigma) for 2 h at 4 °C with gentle rocking. After extensive washing with lysis buffer, proteins bound to the beads were either subjected to in vitro kinase assays (see below) or directly solubilized by boiling in SDS sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and blotted with the indicated antibodies. The reactive bands were visualized with horseradish peroxidase coupled to the appropriate secondary antibodies and the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech).

In Vitro Kinase Assays—Washed immunoprecipitates were resuspended in 20 μl of kinase buffer (20 mM Tris, pH 7.6, 1 mM dithiothreitol, 20 mM MgCl2, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 1 mM EDTA, 1 mM sodium orthovanadate, protease inhibitors (complete, Roche Molecular Biochemicals), 20 μM ATP) with (for autodigestion) or without (for Western analysis) 10 μCi of [γ-32P]ATP and incubated for 20 min at 37 °C. The kinase reaction was stopped by boiling in 20 μl of SDS sample buffer. Proteins were separated via SDS-polyacrylamide gel electrophoresis. The gels were either immunoblotted as described above or dried and exposed to x-ray films.

Computational Analysis—Sequences related to human IRAK were identified in the EST data base (dbEST) at the National Center for Biotechnology Information using the TFASTA program of the Wisconsin Package version 9.1 (Genetics Computer Group (GCG), Madison, WI). Pairwise sequence comparisons were performed with the GAP program of the Wisconsin Package and sequence alignments with the ClustalW function of MacVector version 6.0.1 (Oxford Molecular Group).

Results

Identification of IRAK-M as a New Member of the IRAK/Pelle Family—In an attempt to identify new signal transducers in Toll/IL-1R signaling pathways, we searched EST data bases for sequences similar to IRAK. A murine EST sequence (accession number AA840598) was found which encodes polypeptides sharing significant homology with IRAK. A screen of a human phytohemagglutinin-activated peripheral blood leukocyte cDNA library with this EST sequence resulted in the isolation of a full-length cDNA clone that encodes a protein with 596 amino acids and a calculated molecular mass of 68 kDa. Analysis of the deduced protein sequence revealed an NH2-terminal death domain (39) and a central kinase domain, similar to the domain structures of IRAK and IRAK-2 (Fig. 1). The overall sequence similarity shared between the newly identified protein and the existing IRAK-like molecules is between 30 and 40%.

Table I

<table>
<thead>
<tr>
<th>IRAK-M</th>
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Table I—Sequence similarities in the IRAK/Pelle family

Sequence comparisons were performed using the GAP program of the Wisconsin GCG package. Numbers indicate percentage of sequence similarity in protein pairs.

Fig. 2. Expression pattern of IRAK-M mRNA. A, detection of IRAK-M mRNA in adult human tissues. Multiple tissue Northern blots (CLONTECH) were hybridized, under stringent conditions, with a [32P]-labeled cDNA fragment encoding full-length IRAK-M. RNA size markers are indicated in kilobases. B, detection of IRAK-M mRNA in human cell lines. Northern blots containing 20 μg/lane of total RNA isolated from 293, HeLa, HepG2, Jurkat, MRC-5, U937 (untreated, TPA/ionomycin- or LPS-treated), THP-1 (untreated or TPA/ionomycin-treated) were probed with 32P-labeled cDNA for full-length IRAK-M (top panel), IRAK (middle panel), or IRAK-2 (bottom panel). The positions of 18 S and 28 S rRNA are indicated on the right.
Expression Pattern of IRAK-M mRNA—Hybridization of multiple tissue Northern blots with a [32P]-labeled full-length IRAK-M cDNA revealed three different mRNA species, one of 8 kilobases (Fig. 2A). The size of the smallest RNA species matches the cDNA isolated in the library screen, whereas the nature of the 8-kilobase hybridizing bands is currently unknown. IRAK-M mRNA is detected predominantly in peripheral blood leukocytes and only in low amounts in other tissues. No message was detectable in brain, liver, thymus, and small intestine. A Northern analysis of different cell lines indicates that unlike IRAK, which is expressed in all cell types examined, the expression of IRAK-M is limited to the monocytic cell lines U937 and THP-1. The expression of IRAK mRNA in THP-1 cells is enhanced significantly by TPA and ionomycin treatment, which induces the differentiation of THP-1 toward more mature macrophages. This suggests a possible role of IRAK-M in the differentiation of THP-1 toward more mature macrophages. A Northern analysis of different cell lines indicates that unlike IRAK, which is expressed in all cell types examined, the expression of IRAK-M is limited to the monocytic cell lines U937 and THP-1. The expression of IRAK mRNA in THP-1 cells is enhanced significantly by TPA and ionomycin treatment, which induces the differentiation of THP-1 toward more mature macrophages. This suggests a possible role of IRAK-M in the differentiation of THP-1 toward more mature macrophages.

NF-κB Activation by IRAK Family Members—It was shown previously that overexpression of either IRAK or IRAK-2 activates NF-κB and that overexpression of the death domain-containing NH2 terminus of either protein has a dominant negative effect on IL-1-induced NF-κB activation (19, 20, 40). To investigate the ability of IRAK-M to activate NF-κB, we transiently transfected 293 cells stably expressing IL-1RI (293IL-1RI cells; 21) with different amounts of IRAK-M expression plasmids together with an NF-κB-dependent luciferase reporter construct (Fig. 3A). Overexpression of IRAK-M activated NF-κB in a dose-dependent fashion. The potency and efficacy of IRAK-M in this assay are similar to IRAK-2 but lower than IRAK (for a comparison of expression levels, see Fig. 4 and 5, bottom panels).

Overexpression of the death domains of all three IRAK proteins inhibited IL-1-induced NF-κB activation, yet had a minimal effect on the 10-fold TNF-induced NF-κB activation (Fig. 3B). The NH2 terminus of IRAK was most potent: 3 ng of transfected plasmid reduced IL-1-induced luciferase activity by 50%. 100 ng of IRAK-2 or 400 ng of IRAK-M was needed to obtain the same effect, although the expression levels of the three constructs were similar (data not shown). The similarity shared by the three proteins in the NF-κB reporter assays provides functional confirmation that IRAK-M is a member of the IRAK family. Interaction of IRAK Family Members with MyD88 and TRAF6—IRAK exerts its function in IL-1 signaling by interacting with the upstream adaptor protein MyD88 and the downstream signal transducer TRAF6 (20, 25). IRAK-2 has also been shown to interact with MyD88 and TRAF6 upon overexpression (19). We investigated whether IRAK-M is capable of interacting with the same molecules. Myc-tagged wild type or kinase-inactive forms of IRAK, IRAK-2, or IRAK-M were expressed in 293 cells alone or together with Flag-tagged MyD88 (Fig. 4A). Immunoblotting analysis of the anti-Flag immunoprecipitates showed that mutant IRAK as well as both wild type and mutant IRAK-M and IRAK-2 can associate with MyD88 with a comparable efficiency. Wild type IRAK becomes phosphorylated upon overexpression and does not interact with MyD88.

We then examined if IRAK-M can bind to TRAF6 in a similar transfection experiment. Myc-tagged IRAK-M coprecipitated..
with Flag-tagged TRAF6 in an amount comparable to IRAK
and IRAK-2 (the coprecipitation of IRAK wild type with TRAF6
was omitted because TRAF6 expresses poorly when coex-
pressed with wild type IRAK) (Fig. 4B). The interaction of
IRAK-M with MyD88 and TRAF6 further indicates that it is
able to play a role in signal transduction similar to that of
IRAK and IRAK-2.

Interactions among IRAK Family Members—We investi-
gated the ability of IRAK-M, IRAK, and IRAK-2 to form homo-
or heterocomplexes in a transient transfection assays. Myc-
tagged wild type or kinase-inactive mutants of IRAK, IRAK-2,
and IRAK-M were expressed in 293 cells either alone or to-
gether with their Flag-tagged versions in all possible combina-
tions. Anti-Flag immunoprecipitates of transfected cells were
then immunoblotted to detect coprecipitating Myc-tagged pro-
teins. Although the interactions between wild type IRAK and
any other IRAK proteins were relatively weak because of the
lower levels of expression, all possible hom- and heterocom-
plex formations were readily detectable (Table II), suggesting
that the three IRAK proteins may form complexes in cells
where they are coexpressed.

Analysis of Kinase Activity of IRAK Family Members—IRAK
possess a pronounced kinase activity and can undergo heavy
autophosphorylation after IL-1 stimulation in cells (21, 24). To
compare kinase activities of IRAK-M, IRAK, and IRAK-2, Flag-
tagged wild type or the kinase-defective forms of the three
proteins were transiently expressed in a 293 cell line deficient
for IRAK (293I1A; 41) to avoid coprecipitation of endogenous
IRAK. The epitope-tagged proteins were immunoprecipitated
with anti-Flag M2 beads and tested for their autophosphoryl-
ation activity. Wild type IRAK was heavily phosphorylated, as
indicated by its reduced electrophoretic mobility (Fig. 5, bot-
tom panel) and its strong labeling by [32P]ATP (Fig. 5, top panel). As
expected, IRAK K239S did not undergo autophosphorylation
(Fig. 5). Surprisingly, neither wild type IRAK-M nor wild type
IRAK-2 was able to autophosphorylate like IRAK. After a long
exposure, faint radiolabeled bands with retarded mobility were
detected in the sample containing the wild type IRAK-M (Fig.
5, middle panel), indicating that this protein may have a weak
kinase activity, but we were unable to detect any kinase activ-
ity of IRAK-2.

We then asked if IRAK-M and IRAK-2 can be phosphorylated
and/or activated by IRAK. Flag-tagged wild type or kinase-
defective IRAK-M, IRAK, or IRAK-2 was cotransfected with
untagged IRAK into IRAK-deficient 293I1A cells. Each Flag-
tagged protein coprecipitated the untagged IRAK that under-
got autophosphorylation (Fig. 5, right panel). Coprecipitated
IRAK efficiently phosphorylated IRAK Lys → Ser, as indicated
by the shift of its electrophoretic mobility (bottom panel) and its
heavy [32P] labeling. In comparison, IRAK-M and IRAK-2 are
not as good substrates as IRAK. But it is remarkable that the
wild type IRAK-M and IRAK-2 are phosphorylated more
strongly than their corresponding Lys → Ala mutants, raising
the possibility that IRAK-M and IRAK-2 may be activated by
IRAK and then undergo autophosphorylation.

Restoration of IL-1 Response in IRAK-deficient 293 Cells—It
has been shown recently that the IRAK-deficient 293I1A cells
can no longer respond to IL-1, and this defect can be restored by
IRAK or IRAK-2 (41). To test if IRAK-M can mediate an IL-1
response in the absence of IRAK, we transiently transfected
293I1A cells with different amounts of IRAK-M expression
vector along with a NF-κB-dependent luciferase reporter con-
struct. Similar to IRAK-2, IRAK-M can also supplement for the
loss of IRAK to restore IL-1 response (Fig. 6A). However, nei-
ther IRAK-2 or IRAK-M is as efficacious as IRAK in this assay.
Transfection of 293I1A cells with an expression vector for re-

FIG. 4. Interaction of IRAK family members with MyD88 and TRAF6. A, coprecipitation of MyD88 and IRAK family members. 3 × 10^6 293
cells were transfected with 4 μg of expression plasmid for Myc-tagged IRAK, IRAK-2, or IRAK-M (each wild type and kinase-inactive mutant) alone
or together with 3 μg of Flag-tagged MyD88. After 30 h, cell lysates were prepared and immunoprecipitated (IP) with anti-Flag mAb. Coprecipitating IRAK was detected using anti-Myc antiserum (top panel). Western analysis (WB) of the same blot with mAb to the Flag epitope shows that similar amounts of MyD88 were immunoprecipitated (middle panel). The lysates of the transfected cells were immunoblotted with Myc
antiserum to monitor the expression of IRAK (bottom panel). B, coprecipitation of IRAK proteins and TRAF6. 6.3 × 10^6 293 cells were transfected with Myc-
tagged IRAK, IRAK-2, and IRAK-M with or without 5 μg of expression plasmid for TRAF6. The immunoprecipitation and Western blotting were performed as described in A.
cepto interacting protein, a type I TNF receptor-associated kinase (42), failed to restore the IL-1 response, showing that the IRAK effects were specific (data not shown). These results indicate that the three IRAK-like proteins can at least partially substitute for one another in signaling.

Restoration of LPS Response in IRAK-deficient Cells—TLR-2, a member of the Toll/IL-1R superfamily, has recently been shown to mediate LPS-induced NF-kB activation in 293 cells (9, 12). Because of the sequence similarity shared by the intracellular domains of TLR-2 and IL-1R1/IL-1RaCP, it has been speculated that the intracellular signal transducers for IL-1 might also participate in TLR-2 signaling. Supporting this hypothesis, the IRAK-deficient 293I1A cells that fail to respond to IL-1 also fail to mount a TLR-2-mediated LPS response (Fig. 6B). Transfection of expression plasmids for any of the three IRAK proteins regenerated the LPS response in the luciferase reporter assay (Fig. 6B), again suggesting that these proteins share similar functional properties. This IRAK-mediated LPS response is dependent on TLR-2, as 293I1A transfected with IRAK failed to respond to LPS in the absence of TLR-2 (data not shown).

DISCUSSION

Members of the Toll/IL-1R family of transmembrane receptors play an important role in various immune responses (3, 5, 9, 11–13). Supported by both biochemical and genetic studies, many members of this receptor family engage similar intracellular signal transducers to generate cellular response (see the Introduction). In this study, we present for the first time genetic evidence for the involvement of IRAK in LPS signaling. The IRAK-deficient cell line 293I1A failed to respond to IL-1 as well as to LPS, and this defect can be corrected by introducing exogenous IRAK or IRAK-related proteins back into the cells. These results support an emerging scheme that the IL-1 intracellular signaling mechanism may be common to a class of receptors that share sequence homology with the IL-1 receptors.

The observation that IRAK-deficient mice and cells retain significant residual IL-1 and IL-18 response (33, 34) implicates the presence of molecule(s) with overlapping functions in animals. The likely candidates for such molecules are IRAK-2 and IRAK-M, which share similarities in protein sequence as well as functional properties with IRAK. Compelling support for this notion came from the results that either IRAK-2 or IRAK-M could reconstitute IL-1 and LPS responses in the IRAK-deficient 293 cells. Also in agreement with the role of IRAK-2 and IRAK-M as signaling molecules with functions similar to those of IRAK, IRAK-deficient fibroblasts retained partial response to IL-1 (33), whereas the IL-1 and LPS response is essentially abolished in IRAK-deficient 293 cells, which lack IRAK-M and express low levels of IRAK-2.

The three IRAK-like proteins are unlikely to play completely redundant roles in animals. In contrast to IRAK and IRAK-2, whose mRNA is detected in multiple tissues and cell types, the expression of IRAK-M is limited mostly to cells of the monocytic lineage and is up-regulated during differentiation, suggesting a cell type-specific function of IRAK-M.

The most striking feature that distinguishes IRAK from the other IRAK-like proteins is its potent autophosphorylation, which is not detected in IRAK-M and IRAK-2. Nevertheless, both IRAK-M and IRAK-2 are able to transduce IL-1- and LPS-mediated signals in the absence of IRAK. Assuming that autophosphorylation reflects the kinase activity, it is possible that the role of IRAK in signal transduction is not phosphorylating downstream targets. In nontransfected cells, IRAK autophosphorylation is detected only after IL-1 stimulation. Based on the observation that the phosphorylated form of IRAK is unable to bind to MyD88, which links IRAK to the receptor complex (20), we speculated that the phosphorylation of IRAK...

![Fig. 5. Analysis of IRAK kinase activity.](http://www.jbc.org/)

**TABLE II**

<table>
<thead>
<tr>
<th>Homo and hetero association of IRAK family members</th>
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<tr>
<td>293 cells (3 × 10⁶) were cotransfected with 4 µg of expression plasmids for Flag-tagged and Myc-tagged wild type (wt) or kinase-inactive forms of IRAK-M, IRAK, and IRAK-2 as indicated. After 30 h, cell lysates were prepared and immunoprecipitated with anti-Flag mAb. Coprecipitating Myc-tagged IRAK was detected using a Myc antiserum. Western analysis of the same blot with anti-Flag mAb shows that similar amounts of Flag-tagged IRAK were precipitated. The lysates of the transfected cells were immunoblotted with Myc antiserum to monitor the equal expression of the Myc-tagged IRAK constructs. Weak interactions were depicted by a single +, strong interactions by +++.</td>
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<tr>
<td>IRAK-M wt</td>
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<td>IRAK-M wt</td>
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<td>IRAK-2 wt</td>
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<td>IRAK-2 K237A/K238A</td>
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may trigger its release from the receptor complex and its subsequent interaction with TRAF6. If this assumption were correct, the requirement of the IRAK kinase activity in signaling would not be apparent in transfection experiments because of protein overexpression, which results in a high concentration of free IRAK. Consistent with this scenario, forms of IRAK proteins harboring mutations in their ATP binding pockets could still activate NF-kB upon overexpression in 293 cells, albeit with a lower potency (data not shown). Alternatively, the role of IRAK kinase activity may be to regulate IL-1 signaling negatively after cytokine induction. Supporting this hypothesis, IRAK phosphorylation is followed by its proteolytic degradation (24). Careful comparison of the duration of the IL-1 response in cells expressing physiological amounts of the wild type IRAK or the kinase-deficient form of IRAK will help to distinguish between these possibilities.

Although IRAK-2 and IRAK-M have very low autophosphorylation activity on their own, the wild type forms can be phosphorylated better than their corresponding kinase-inactive mutants in the presence of IRAK (Fig. 5). Under the assumption that the mutation of a single amino acid residue in the ATP binding pocket does not transform IRAK-M or IRAK-2 into a poorer substrate for IRAK, it is reasonable to speculate that IRAK phosphorylates IRAK-2 and IRAK-M, thereby activating the intrinsic kinase activity of these molecules. Mapping the sites on IRAK-2 and IRAK-M which are phosphorylated by IRAK in combination with site-directed mutagenesis should help to address this issue.

Our study shows that IRAK is more potent and efficacious than the other two IRAK-like proteins at restoring IL-1 and LPS responses in 293I1A cells. Although we cannot exclude that IRAK-M and IRAK-2 mainly participate in the signaling of other related receptors, our observations raise the possibility that IRAK may be the primary signal transducer. Because IRAK-2 and IRAK-M can form heterocomplexes with IRAK, they may help to amplify the cytokine signals by providing a critical mass. Under this scenario, the expression levels of IRAK-like molecules would influence the degree of the response of a given cell type to a set of cytokines such as IL-1, IL-18, or LPS.

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IRAK-M Is a Novel Member of the Pelle/Interleukin-1 Receptor-associated Kinase (IRAK) Family
Holger Wesche, Xiong Gao, Xiaoxia Li, Carsten J. Kirschning, George R. Stark and Zhaodan Cao

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