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Regulation of IRAK-4 kinase activity via autophosphorylation within its activation loop

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Abstract

Interleukin-1 stimulation leads to the recruitment of MyD88, interleukin-1 receptor-associated kinase 1 (IRAK-1) and interleukin-1 receptor-associated kinase 4 (IRAK-4) to the IL-1 receptor. The formation of the IL-1 receptor complex triggers a series of IRAK-1 autophosphorylations, which result in activation. IRAK-4 is upstream of IRAK-1 and may act as IRAK-1 kinase to transmit the signal. To date, there is no upstream kinase reported for IRAK-4; the activation mechanism of IRAK-4 remains poorly understood. Here, for the first time, we report three autophosphorylation sites that are responsible for IRAK-4 kinase activity. LC–MS/MS analysis has identified phosphorylations at T342, T345, and S346, which reside within the activation loop. Site-directed mutants at these positions exhibit significant reductions in the catalytic activity of IRAK-4 (T342A: 57%; T345A: 66%; S346A: 50%). The absence of phosphorylation in kinase-dead IRAK-4 indicates that phosphorylations in the activation loop result from autophosphorylation rather than from phosphorylation by an upstream kinase. Finally, we demonstrate that autophosphorylation is an intramolecular event as wild-type IRAK-4 failed to transphosphorylate kinase-inactive IRAK-4. The present data indicate that the kinase activity of IRAK-4 is dependent on the autophosphorylations at T342, T345, and S346 in the activation loop.
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Keywords: IRAK-4; Activation loop; Autophosphorylation; Kinase activity; Kinetics; Mutation; Phosphorylation site

The IL-1 receptor-associated kinases (IRAKs) are central mediators of Toll/IL-1 (TIR) signal transduction. IRAK-1, the first family member, was first reported in 1994 [1] and subsequently cloned and characterized in 1996 [2]. Upon ligand stimulation, IRAK1 is rapidly recruited to the receptor, resulting in the assembly of the receptor complex and subsequent downstream signaling via NF-κB and AP-1 [3–6]. IL-1 response in IRAK-1 deficient mice is impaired but not abolished, and this effect has been attributed to redundancy in the IRAK family [7]. To this end, two additional family members have been identified in humans, IRAK-2 and IRAK-M, which can compensate for the loss of IRAK-1 in mutant 293 cells. Unlike IRAK-1, however, both proteins lack kinase activity; several reports have indicated that the kinase activity of IRAK-1 may be dispensable for downstream signaling through NF-κB [6,8].

Interleukin-1 receptor-associated kinase 4 (IRAK-4), the latest member of the IRAK family, was reported in 2002 [9]. It is known that IRAK-4 plays a pivotal role in

Abbreviations: IL-1R, interleukin-1 receptor; TIR, Toll/IL-1 plant R-gene; IRAK, interleukin-1 receptor-associated kinase; NF-κB, nuclear factor κB; AP-1, activating protein-1; MyD88, a cytoplasmic adaptor protein that contains a TIR domain; *ip*, immunoprecipitation; *wt*, wild-type; *kd*, kinase dead; DTT, dithiothreitol.

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mediating the signal transduction of the TIR signaling pathway as both IRAK-4 knockout mice and human patients with inherited IRAK-4 deficiency exhibit functional defects in this pathway [9,10]. Unlike IRAK-2 and IRAK-M, IRAK-4 (also IRAK-1) exhibits potent catalytic kinase activity. Overexpression of IRAK-4 activates NF-κB and MAPK pathways in a kinase-activity dependent manner, and dominant-negative IRAK-4 effectively inhibits IL-1-induced NF-κB activation [9]. Failure to recruit IRAK-4 to the IL-1 receptor complex leads to inhibition of IRAK-1 hyperphosphorylation subsequent to activation [11]. Although the capability of IRAK-4 to phosphorylate IRAK-1 *in vitro* [9,11] has led some researchers to believe that IRAK-4 mediates TIR signaling by acting as an IRAK-1 kinase, recent experiments with kinase-inactive IRAK-4 have cast doubt upon this conclusion. Indeed, kinase-inactive IRAK-4 has the same ability as wild-type IRAK-4 in restoring IL-1 mediated signaling in human IRAK-4-deficient cells, including NF-κB and JNK activation, NF-κB-dependent gene expression, and IL-8 gene expression [12]. Taken together, the role of IRAK-4 kinase activity in TIR signaling remains controversial. However, as the impairment of the kinase activity of both IRAK-1 and IRAK-4 efficiently abolishes the IL-1 pathway [12], it is likely that the combination of IRAK-1 and IRAK-4 kinase activities is important for signaling.

The molecular mechanism of IRAK-1 hyperphosphorylation subsequent to activation is fairly well documented in the literature [12]. Upon ligand stimulation, IRAK-4 and IRAK-1 are recruited to IL-1R/TLRs complex by MyD88. IRAK-1, in turn, undergoes hyperphosphorylation initiated by either autophosphorylation or phosphorylation by IRAK-4; hyperphosphorylated IRAK-1 then dissociates from the receptor complex and is eventually degraded. The mechanism of IRAK-4 activation, on the other hand, has not been reported. Here we sought to examine phosphorylation events within the IRAK-4 activation loop and found that overexpressed IRAK-4 is activated via intramolecular phosphorylation in its activation loop.

Experimental

General materials. Reagents used for cloning, expression, purification, and immunoprecipitation, as well as sample preparation for analysis by mass spectrometry are specified within the method description. Biotinylated peptide substrate, biotin-C6-KAKVTGRWKRTSMKLL-amide (Peptide I), was custom synthesized by New England Peptide, Inc. (Gardner, MA). Anti-phospho-IRAK-4 (pT345/pS346) mouse polyclonal antibody was generated by Cell Signaling Technology (antigen: a short peptide around Thr-345 and Ser-346). SAM$^{2®}$ Biotin Capture Membrane (Cat. No. V7861) was purchased from Promega (Madison, WI). [γ-^{33}P]-adenosine 5′-triphosphate (Cat. No. NEG602H001MC) was purchased from Perkin Elmer Life Sciences (Wellesley, MA). Adenosine 5′-triphosphate (Cat. No. 27-2056-01) was purchased from Amersham Biosciences (Piscataway, NJ). Reagents or chemicals not specified were purchased from Sigma. Radioactivity was quantified using a PhosphoImager (FUJIFILM BAS250).

Expression of human recombinant IRAK-4. Human IRAK-4 cDNA was amplified from a human thymus cDNA clone and subcloned into pTracer-EF/V5-His (Invitrogen™, Carlsbad, CA) as a C-terminal V5-$(His)_6$ fusion. The construct with confirmed DNA sequence was then subcloned into a pFastBac N-terminal $(His)_6$-tagged transfer vector (Invitrogen™) expressed in Sf9 cells. The protein expressed in Sf9 cells was extracted from cell lysate using a Ni–NTA affinity resin (Qiagen) and further purified with Superdex 200™ (Pharmacia). The purified proteins were stored in Buffer A (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% v/v Tween 20, 10% v/v glycerol, and 2.5 mM β-mercaptoethanol) at −80 °C. Freeze–thaw cycles did not appear to affect the activity. The kinetic properties, including kinetic parameters and inhibition profile of this protein, were comparable to the human full-length C-terminal V5-$(His)_6$ IRAK-4 protein that was immunoprecipitated from HEK293T cells which overexpress the protein (data not shown). This suggests that the N-terminal $(His)_6$ tags had no effect on the catalytic integrity of IRAK-4.

Site-directed mutagenesis. All of the site-directed mutants, K213A/K214A (kinase dead), T342A, T345A, T346A, T345A/S346A, and T342A/T345A/S346A, were generated using overlapping extension PCR [13]. First, a C-terminal 630 bp fragment was amplified using inner primers containing the desired mutation(s). The N-terminal fragment containing 787 bp was obtained by PCR using wild-type IRAK-4 as a template. The N-terminal and C-terminal fragments containing the overlapping sequences were purified and used as templates for extension PCR to afford the full-length IRAK-4. The outer extension PCR primers were designed to contain an *Eco*RI site prior to the initiation codon and an *Xba*I site after the last amino acid. The final PCR products were digested with *Eco*RI and *Xba*I, and subcloned into the same sites of pTracer-EF/V5-His (A) (Invitrogen™) for expression. The proteins expressed in HEK293T cells were lysed using 1× Kinase Lysis Buffer (Cell Signaling Technology) and stored in Buffer A at −80 °C. Prior to experiments, proteins were freshly immunoprecipitated (see *Immunoprecipitation*).

For *Baculovirus* expression, T342A, T345A, S346A, or T345A/S346A mutants were generated by PCR amplification from the corresponding templates of pTracer-EF/V5-His; T342A/T345A mutant was generated by site-directed mutagenesis from pTracer-EF/V5-His T342A/T345A/S346A. The DNA fragments containing the desired mutation(s) were subcloned into a pFastBac N-terminal $(His)_6$-tagged vector (Invitrogen™). The resulting constructs with confirmed DNA sequences were expressed in Sf9 cells and extracted from cell lysate using a Ni–NTA resin (Qiagen).

Immunoprecipitation. A value of 2×10^6 HEK293T cells were seeded into 100-mm Petri dishes containing DMEM with 10% FCS, 20 mM glutamine diluted from a 10× stock (Cat. No. 25030-081, Invitrogen), and 1× penicillin/streptomycin diluted from a 100× solution (Cat. No. 10378-016, Invitrogen). After reaching 50% confluence (~17 h), the cells were transfected with 6 μg of DNA using a Fugene kit (Cat. No. 1814443, Roche, Indianapolis, IN) and the protocol described within (4 μL/μg DNA in 100 μL medium). After 48 h of culture, the cells were harvested and lysed using the Cell Lysis Buffer (Cat. No. 9803) from Cell Signaling Technology (Beverley, MA). The lysates were vortexed vigorously, incubated on ice for 15 min, and centrifuged at 5000 rpm for 10 min at 4 °C. The resulting clear supernatant was then immunoprecipitated using anti-V5 monoclonal antibody in the presence of fresh Protease Inhibitor Cocktail (Complete, mini EDTA-free, Cat. No. 11836170001, Roche Applied Sciences, Indianapolis, IN) and eluted off the beads using the Gentle Ag/Ab Elution Buffer (Cat. No. 21027, PIERCE, Rockford, IL). The eluted protein solution was then passed through a Zeba™ Desalt Spin Column (Cat. No. 89882, PIERCE, Rockford, IL) to exchange into Buffer A and used freshly. However, subsequent experiments showed that freeze–thaw cycles had no effect on the catalytic activity or stability of immunoprecipitated IRAK-4.

Mass spectrometry. Phosphosite mapping of recombinant N-terminally His6 tagged wild-type IRAK-4 (*wt*-IRAK-4) by nano LC–MS/MS was performed on 30 μg of purified, recombinant protein expressed in Sf9 cells following a non-radioactive *in vitro* autophosphorylation reaction (see *In vitro autophosphorylation of IRAK-4*). In order to maximize amino acid coverage of IRAK-4, and more importantly, the recovery of pS- and pT-containing phosphopeptides, the *wt*-IRAK-4 was digested directly on Ni–

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