

# Threonine 66 in the death domain of IRAK-1 is critical for interaction with signaling molecules but is not a target site for autophosphorylation

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**Abstract:** Ligand binding in the TLR/IL-1R family results in the transient formation of an intracellular signaling complex, which contains, amongst others, the serine/threonine-specific kinase IL-1R-associated kinase 1 (IRAK-1). Concomitantly, the kinase function of IRAK-1 becomes activated, resulting in massive autophosphorylation and finally in the dissociation of the initially constituted signaling complex. The death domain (DD) of IRAK-1 mediates the interaction with other molecules of the signaling complex, e.g., the adaptor MyD88, the silencer Tollip, and the activator kinase IRAK-4. The conserved threonine at position 66 (T66), located within the DD, is a putative autophosphorylation target site. Here, we provide evidence that T66 critically impacts the secondary structure of the IRAK-1 DD. Thereby, it ensures the transient manner of interactions between IRAK-1 and the other signaling molecules. This essential role, however, is not regulated by phosphorylation of T66 itself. *J. Leukoc. Biol.* 84: 807–813; 2008.

**Key Words:** inflammation · kinases · interleukin-1 · Toll-like receptor · Tollip

## INTRODUCTION

Recognition of molecular patterns of microbial or viral origin by TLR on sentinel cells is an essential step in the activation of the innate as well as the adaptive immunity of the host [1, 2]. Upon activation, sentinel cells rapidly release proinflammatory cytokines including IL-1 $\beta$  and IL-18, which orchestrate the acute inflammatory response. TLR and IL-1R family members share a highly homologous domain in their intracellular part, the TLR/IL-1R (TIR) domain, justifying that these receptors are subsumed in the TIR family.

Upon activation of a TIR family member (with the exception of TLR3), the cytosolic serine/threonine kinases IL-1R-associated kinase 1 (IRAK-1) and IRAK-4, members of a small family of specialized adaptor molecules [3, 4], are recruited to the receptor intracellular TIR domains via the adaptor MyD88 [5]. Subsequently, TNFR-associated factor 6 (Traf6) is acti-

vated by binding to IRAK-1, resulting in the activation of MAPK cascades and NF- $\kappa$ B. Finally, the expression of a specific target gene profile is induced.

In unstimulated cells, IRAK-1 is associated with the silencer Tollip [6, 7]. Recently, we demonstrated that the association of IRAK-1 with Tollip as well as with MyD88 and IRAK-4 is mediated by the death domain (DD) of IRAK-1 [8]. Threonine 66 (T66) within the DD of IRAK-1 is a highly conserved amino acid, found in a series of DD-containing proteins. Mutation of IRAK-1 T66 into alanine (T66-A) was reported to reduce the autophosphorylation of IRAK-1 [9] and to enable stable binding of Tollip [6]. This was regarded a regulatory mechanism, driven by the phosphorylation of T66, which is denied in IRAK-1 T66-A [6, 10]. However, replacing T66 by phosphomimetic amino acids (T66-D, T66-E) resulted in stable binding of adaptor proteins as well [8, 9], contradicting the above-mentioned interpretation. This led us to analyze the putative phosphorylation of IRAK-1 at position T66 in a detailed manner. Here, we demonstrate that T66, in its native status, is critical for the maintenance of the secondary structure of the DD of IRAK-1 and thus, for the interaction of IRAK-1 with other signaling components. The phosphorylation of T66, which might be a regulatory mechanism for these interactions, however, could not be detected.

## MATERIALS AND METHODS

### Cell culture and biological reagents

Human embryonal kidney (HEK)293RI cells, which stably overexpresses the type I IL-1R, were grown in DMEM supplemented with 10% (v/v) FCS and 2 mM L-glutamine at 37°C and 10% CO<sub>2</sub>. The mouse thymoma cell line EL-4 6.1 was maintained in RPMI medium supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 1 mM pyruvate, and 1 $\times$  nonessential amino acids. Anti-Flag reagents were from Sigma-Aldrich (St. Louis, MO, USA), the anti-myc antibody (9E10) was from Santa Cruz Biotechnology (Santa Cruz, CA,

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USA), and rat anti-Tollip antibody (Kimmy-1) was from Alexis Biochemicals (San Diego, CA, USA). A mAb against human IRAK-1 (2A9) was kindly provided by Zhaodan Cao (Amgen, South San Francisco, USA). Recombinant human (rh)IL-1 $\beta$  was kindly provided by Diana Boraschi (Dompe SpA, L'Aquila, Italy).

## Expression vectors and cloning procedures

The expression vectors encoding human wild-type (w.t.) IRAK-1 (pRK5-IRAK) or kinase-inactive IRAK-1 (IRAK K239S) were kind gifts of Z. Cao (Amgen) and have been described elsewhere [11]. Plasmids encoding N-terminally Flag-tagged, full-length IRAK-1 (pF1-IRAK-1 f.l., aa 2–712) and the death domain (DD) fragments thereof [pF1-IRAK-1 DDlong (DDL): aa 2–148; pFL-IRAK-1 DDshort (DDS): aa 2–109] were constructed by inserting PCR-generated cDNA fragments into the mammalian expression vector pcDNA3-Flag (kind gift of Bernard Lüscher, Aachen University, Aachen, Germany). The 5 $\times$  NF- $\kappa$ B-luciferase reporter construct was kindly provided by Werner Falk (University of Regensburg, Regensburg, Germany) and has been described elsewhere [12]. The EGFP-IRAK-DDs-TDimer2 fusion plasmid was constructed by replacing the cameleon sequence from plasmid pEGFP-cameleon-TDimer2 (kind gift of Tao Xu, Huazhong University of Science and Technology, Wuhan, China [13]) by PCR-amplified IRAK-1 DDs. This yielded a plasmid coding for the IRAK-1 death domain (aa 2–109), tagged at its N-terminus with EGFP (green fluorescence) and at its C-terminus with TDimer2 (red fluorescence). Point mutations were generated using the Quick Change Mutagenesis Kit (Stratagene) essentially as described by the manufacturer. The deletion mutant of IRAK-1 lacking the ProST region was generated by PCR using the Flag-tagged full-length IRAK-1 plasmid as template and primers substituting the proline-serine-threonine rich region of IRAK-1, aa 110211 (ProST) sequence by a hemagglutinin sequence. The cDNA coding for Tollip was amplified from a human library and ligated into the pcDNA3 vector (Invitrogen). All resulting plasmids were checked by sequencing.

## NF- $\kappa$ B reporter gene assay and quantification of IL-2 production

EL-4 6.1 cells ( $5 \times 10^6$ ) were transfected with differing amounts of IRAK-1 expression plasmids as indicated in the figures. For detection of NF- $\kappa$ B-dependent reporter gene activity, 0.5  $\mu$ g 5 $\times$  NF- $\kappa$ B plasmid was cotransfected. After 4 h, cells were left untreated or were stimulated with 10 ng/ml rhIL-1 $\beta$ . For quantification of IL-2 production, 250 nM A23187 ionophore (Sigma-Aldrich) was added to the cultures. Twenty hours later, cells (NF- $\kappa$ B activity) or supernates (IL-2 production) were harvested. NF- $\kappa$ B activity was measured in cellular lysates using the Luciferase assay system (Promega, Madison, WI, USA) and normalized on the basis of protein concentration measured by protein assay (Bio-Rad, Hercules, CA, USA). IL-2 concentrations in cell supernatants were quantified by ELISA, using the mouse IL-2 development kit (R&D Systems, Minneapolis, MN, USA). Means  $\pm$  SD within the experimental groups were calculated, and statistical significance of differences between experimental groups was evaluated using Student's *t*-test. *P* values <0.05 (\*) and <.01 (\*\*) were considered significant.

## Immunoprecipitation and immunoblotting

HEK293RI cells ( $2 \times 10^6$ ) were seeded in 100 mm petri dishes and transfected the following day by the calcium phosphate precipitation method. The amounts of coding plasmid differed depending on the assay and the constructs used. The total amount of DNA was kept constant in each transfection by adding empty vector to a total of 10  $\mu$ g/dish. Sixteen hours after addition of the DNA precipitates to the cells, the medium was changed. The following day, cells were collected and washed in PBS. Lysis, immunoprecipitation, and immunoblotting were performed as described elsewhere [14, 15]. In brief, cells were lysed by rotation for 30 min at 4 $^{\circ}$ C in buffer containing 0.5% (v/v) Nonidet P-40, 50 mM Hepes (pH 7.9), 250 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 20 mM  $\beta$ -glycerophosphate, 5 mM p-nitrophenylphosphate, and 1 mM Na-orthoovanadate (all from Sigma-Aldrich) and 1 $\times$  complete protease inhibitor mix (Roche, Mannheim, Germany), and debris was removed by centrifugation (10 min, 15,000 *g*, 4 $^{\circ}$ C). Flag-tagged IRAK-1 proteins were immunoprecipitated with the anti-IRAK antibody 2A9 and protein-G sepharose (Amersham, Piscataway, NJ, USA) or with M2-agaarose, recognizing the Flag-tag. Immunoprecipitated proteins were washed, eluted from the sepharose/agaarose by 10 min heating at 95 $^{\circ}$ C in Laemmli buffer, separated by SDS-PAGE, and elec-

troblotted onto polyvinylidene difluoride membranes, which were blocked in 5% dry-milk powder in TBS containing 0.1% Tween 20 for at least 1 h. Specific proteins were detected using anti-Flag (Bio-M2), anti-myc, and anti-Tollip reagents together with the appropriate HRP-coupled secondary reagents and ECL.

## In vitro kinase assay and MALDI mass spectrometry

For in vitro kinase assay, HEK293RI cells were transfected, and resulting proteins were immunoprecipitated. Washed immunoprecipitates were incubated in kinase buffer (20 mM Hepes, pH 6.5, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 1  $\mu$ M ATP) in the presence of 1  $\mu$ Ci <sup>32</sup>P- $\gamma$ ATP for 30 min at 37 $^{\circ}$ C. The reaction was stopped by 10 min heating at 95 $^{\circ}$ C in Laemmli buffer, and the proteins were separated by SDS-PAGE. Gels were dried and exposed to X-ray films (BioMax, Kodak, Rochester, NY, USA).

For mass spectrometric analysis, cells were transfected with the indicated plasmids and stimulated for 20 min with 10 ng/ml IL-1 $\beta$  prior to lysis. Proteins were immunoprecipitated, either or not subjected to in vitro kinase assay, reduced, and treated with iodoacetamide. Subsequently, they were separated by SDS-PAGE and visualized by Coomassie staining. The bands containing the proteins of interest (Fl-IRAK-1 f.l.:  $\geq 100$  kDa; Fl-IRAK-DDL: 18 kDa; Fl-IRAK-1-DDs: 13 kDa) were excised and digested in-gel with trypsin by a standard procedure. Resulting peptides were detected in an Ultraflex mass spectrometer (Bruker Daltonics, Billerica, MA, USA) in linear or reflector mode using  $\alpha$ -cyanohydroxy cinnamic acid as matrix.

## Fluorescence resonance energy transfer (FRET) analysis

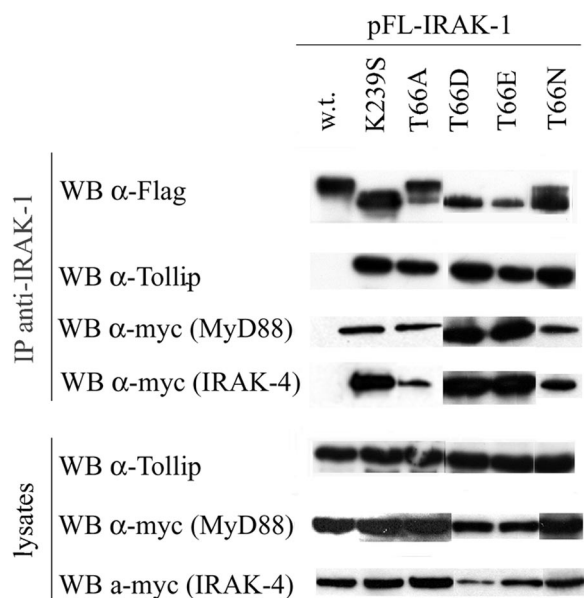
For intramolecular FRET analysis, HEK293RI cells were grown on glass coverslips and transfected the following day with EGFP-IRAK-DDs-TDimer2 fusion plasmids as described above. Forty-eight hours after transfection, cells were washed in PBS and fixed in buffered formalin. Confocal imaging was done on a Leica DM inverted research microscope equipped with a TCS SP2 Acousto-Optical beam splitter scanhead. Fluorescent proteins were excited at 458 nm (EGFP) or at 543 nm (TDimer2), and emission was collected within the bands of 505–535 nm and 605–655 nm for EGFP and TDimer2 fluorescence, respectively. FRET was analyzed and calculated using Leica confocal software.

## RESULTS

### Interaction of IRAK-1 with adaptor molecules

IRAK-1 can be activated by TIR ligand-induced signaling or by ectopic expression of exogenous IRAK-1, resulting in massive autophosphorylation and subsequently, in a reduced electrophoretic mobility in SDS-PAGE [14, 11]. Additionally, the phosphorylation of IRAK-1 leads to the loss of its stable interactions with signaling molecules such as Tollip, MyD88, and IRAK-4. These interactions are mediated by the DD of IRAK-1 [6, 8], including the amino acid residue T66, which is highly conserved among DD-containing proteins. It was speculated that T66 of IRAK-1 is a target for phosphorylation, regulating the interaction with signaling molecules [6, 10], probably via alteration of the conformation of the DD [16].

This assumption could be tested by coimmunoprecipitation experiments, which detect stable interactions between two molecules. Thus, coimmunoprecipitation of overexpressed IRAK-1 and signaling molecules, which is not detectable when using native, autophosphorylating, w.t. IRAK-1, should be enabled by introducing amino acid residues at position 66, which are usually neither phosphorylated nor mimic phosphorylation (T66-A, T66-N). On the other hand, such coimmunoprecipitation should still be undetectable after mutation of



**Fig. 1.** Mutation of T66 of IRAK-1 enables coimmunoprecipitation of Tollip, MyD88, and IRAK-4. Flag-tagged IRAK-1 constructs, as indicated above the figure, were expressed in HEK293RI cells in combination with untagged Tollip, myc-tagged MyD88, or myc-tagged IRAK-4. IRAK-1 proteins were immunoprecipitated (IP) with the anti-IRAK-1 antibody 2A9, and coimmunoprecipitated Tollip, MyD88, and IRAK-4 were detected by Western blotting (WB) using Tollip- and myc-specific reagents. Efficient immunoprecipitation of IRAK-1 molecules was verified by anti-Flag Western blot, and expression of Tollip, MyD88, and IRAK-4 was checked by Western blotting of whole cell lysates (lysates).

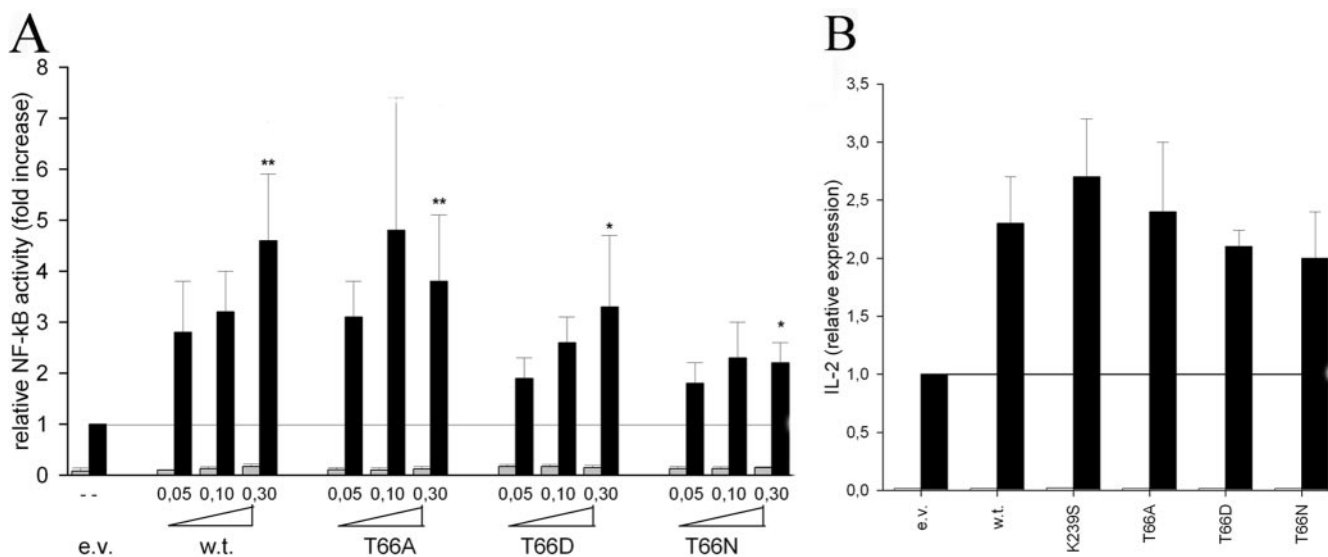
IRAK-1 T66 into phosphomimetic amino acid residues (T66-D, T66-E). Performing such experiments, we found, to our surprise, that all the mutations in IRAK-1 mentioned above allowed efficient coimmunoprecipitation of Tollip, MyD88, and IRAK-4, as it did the kinase-inactivating mutation K239S (**Fig. 1**). Thus, the regulating function of IRAK-1 T66 seems to be at least questionable.

The signaling capacity of IRAK-1 was not abolished by introduction of the kinase-inactivating mutation K239S [12]. To test the effect of mutating T66 in IRAK-1 on signaling, NF- $\kappa$ B activation and IL-2 production, upon transient transfection and IL-1 stimulation, were analyzed. Ectopic expression of w.t. IRAK-1 or T66 mutants enhanced IL-1-induced NF- $\kappa$ B activity in a dose-dependent manner (**Fig. 2A**) as well as IL-2 production (Fig. 2B). However, the effects of transient expression of w.t. IRAK-1 on the one hand and T66 mutated forms of IRAK-1 on the other hand on IL-1-induced signaling did not differ significantly. Thus, similar to the K239S mutation, none of the T66 mutations deactivated the signaling function of the resulting IRAK-1 molecules.

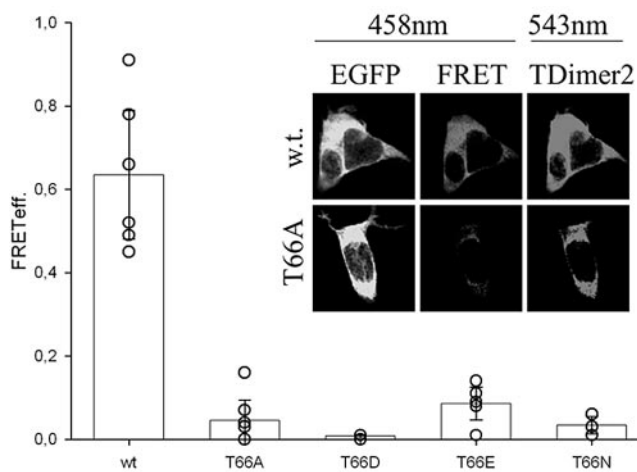
These data show that the amino acid T66 in IRAK-1 denies stable interaction with the signaling molecules. They also suggest that phosphorylation of T66 is not the mechanism disabling interaction of IRAK-1 with the signaling molecules, raising the questions of whether all mutations introduced at position T66 alter the conformation of the DD and whether T66 indeed is phosphorylated in activated IRAK-1.

#### Mutation of T66 alters IRAK-1-DD structure

To test whether possible alterations of the conformation of the DD depend on mutation of T66, we performed intramolecular



**Fig. 2.** Mutation of T66 in IRAK-1 does not inhibit the function of IRAK-1 in IL-1-induced signaling. (A) EL-4 6.1 cells were transfected with increasing amounts of pFL-IRAK-1 constructs as indicated (e.v.=empty vector pcDNA3FL) together with 0.5  $\mu$ g of a NF- $\kappa$ B-dependent luciferase reporter gene construct. After 20 h of incubation in the absence (gray bars) or presence (black bars) of 10 ng/ml IL-1 $\beta$ , NF- $\kappa$ B activity was analyzed in cellular lysates and normalized to total cellular protein content. For presentation, the normalized NF- $\kappa$ B activities of empty vector-transfected and IL-1 $\beta$ -stimulated cells were set to 1, and relative NF- $\kappa$ B activities were calculated on this basis. Shown are means  $\pm$  SD of two to six experiments. Statistical significant differences are indicated (\*,  $P < 0.05$ , vs. e.v.; \*\*,  $P < 0.01$ , vs. e.v.). (B) EL-4 6.1 cells were transiently transfected with 0.3  $\mu$ g pFL-IRAK-1 constructs as indicated. After 20 h of incubation in the presence of 250 nM A23187 (gray bars) or of 10 ng/ml IL-1 $\beta$  + 250 nM A23187 (black bars), IL-2 concentrations were measured in cell supernates. The IL-2 concentrations in supernates of empty vector-transfected cells were set to 1, and relative IL-2 concentrations were calculated on this basis. Shown are means  $\pm$  SD of two experiments.



**Fig. 3.** Mutation of IRAK-1 T66 alters the conformation of the DD. Shown are FRET efficiencies (eff) analyzed by confocal imaging of HEK293RI cells expressing EGFP-IRAK-1-DDs-TDimer2 proteins as indicated on the x-axis. The inset displays representative photographs demonstrating high (w.t.) and low (T66-A) FRET efficiencies.

FRET analysis, indicative of the relative distances between N- and C-termini of the tested proteins: w.t. and T66-mutated DD fragments. As demonstrated in **Figure 3**, mutation of T66 altered the effective FRET in comparison with the w.t. DDs construct, independently of the amino acid introduced (-A, -D, -E, or -N). Thus, the T66 itself critically impacts the structure of the IRAK-1 DD. As T66 replacement by phosphomimetic amino acids and amino acids, which usually cannot become phosphorylated, gave identical results, it seems that the conformational alteration is not regulated by phosphorylation of T66.

### Apparent molecular weight and in vitro autophosphorylation of IRAK-1 T66 mutants

Stable interaction of IRAK-1 with the signaling molecules, thus the interaction-enabling DD conformation, is achieved by the absence of phosphorylation in IRAK-1 (IRAK-1 K239S). It was postulated that substitution of T66 by alanine denies autophosphorylation of the mutant molecule [9]. To verify this theory, we overexpressed the IRAK-1 mutants and performed Western blot analyses of whole cell lysates and immunoprecipitated IRAK-1 molecules (**Fig. 4**, top and middle panels, respectively). The IRAK-1 proteins T66-D and -E migrated similarly to the kinase-inactive mutant K239S (~80 kDa). The mutants T66-A and -N display two main protein species migrating at ~80 kDa and ≥100 kDa, thereby showing a mixed phenotype of the K239S (~80 kDa) and w.t. (≥100 kDa) IRAK-1 molecules. Thus, if using the reduced electrophoretic mobility as indication, these data would suggest that mutation of T66 at least partly abolishes autophosphorylation of IRAK-1.

To experimentally prove this assumption, we subjected the immunoprecipitated IRAK-1 molecules to an in vitro kinase assay (**Fig. 4**, bottom panel). As expected, IRAK-1 w.t. readily autophosphorylated, and this function was lost in the kinase-inactive mutant K239S. Mutation of T66, in contrast, although altering the electrophoretic mobility in SDS-PAGE, did not abolish autophosphorylation of the resulting molecules.

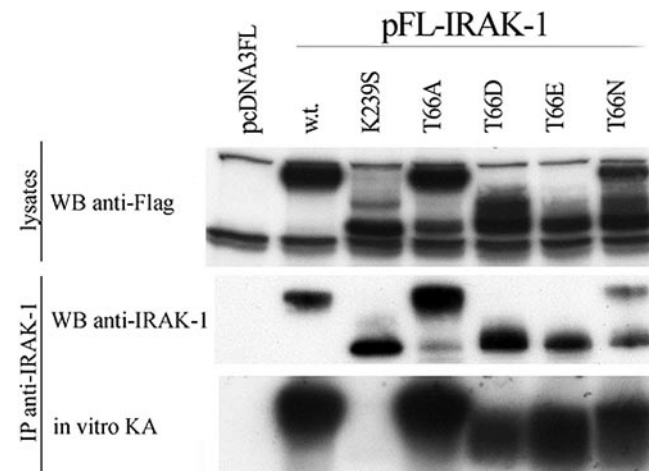
Together, these data indicate that autophosphorylation of IRAK-1 molecules is not denied by mutation of T66. It seems that a modification of the DD, which probably depends on phosphorylation and is mediated by T66, is at the basis for the interaction with signaling molecules and the shift of the electrophoretic mobility in SDS-PAGE. Thus, also in this respect, the question arises of whether T66 in the DD of IRAK-1 is a target for phosphorylation.

### T66 in the overexpressed IRAK-1 DD construct is not phosphorylated

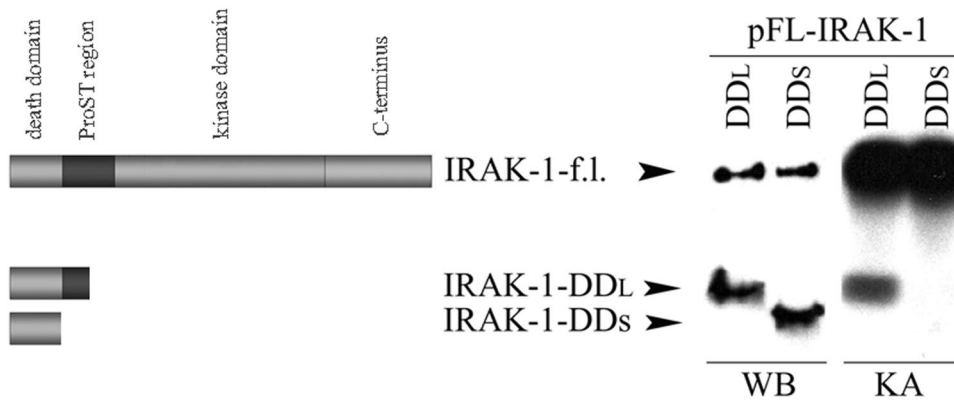
To test phosphorylation at T66 directly, the Flag-tagged IRAK-1 DDs construct and the Flag-tagged kinase active IRAK-1 w.t., full-length molecule were coexpressed, immunoprecipitated via their Flag-tag (forced coimmunoprecipitation), and subjected to in vitro kinase assay. As a control, the construct IRAK-1-DDL, which contains the DD and an additional part of the ProST region, was processed in an identical manner. It has been shown previously that in such an assay system, IRAK-1 w.t. was able to phosphorylate kinase-inactive IRAK-1 fragments [14].

Performing this assay, only IRAK-1-DDL but not IRAK-1-DDS became phosphorylated (**Fig. 5**), indicating that phosphorylation occurred within the part of the ProST region [14] but not within the DD. This, however, might be a result of steric reasons, or a kinase different from IRAK-1 may be active on T66 [10]. Therefore, we analyzed the IRAK-1 DD from the IRAK-1-DDS or -DDL proteins or from the full-length molecule (not shown) after ectopic expression and stimulation of the cells with IL-1β by mass spectrometry to detect T66, possibly phosphorylated within the cellular context.

The IRAK-1-DDS protein gave rise to main peaks of positively charged ions with masses corresponding to the tryptic digestion-derived fragments aa 33–51, aa 52–57, aa 62–76, aa 66–76, aa 80–94, aa 95–109, and aa 97–109



**Fig. 4.** IRAK-1 is phosphorylated independently of the mutation of T66. Flag-tagged IRAK-1 constructs as indicated above the figure were expressed in HEK293RI cells. Whole cell lysates were separated by SDS-PAGE and analyzed by Western blot using anti-Flag reagents. IRAK-1 proteins were immunoprecipitated from whole cell lysates using the anti-IRAK-1 antibody 2A9 and thereafter, analyzed by anti-IRAK-1 Western blotting and by in vitro kinase assay (KA).



**Fig. 5.** The IRAK-1 DD construct is not phosphorylated by IRAK-1 in an in vitro kinase assay after forced coimmunoprecipitation [14]. The Flag-tagged IRAK-1 constructs DDs (aa 2–109) and DDL (aa 2–148) were expressed in HEK293RI cells in combination with Flag-tagged, full-length IRAK-1. Flag-tagged molecules were coimmunoprecipitated using anti-Flag (M2) agarose, and resulting precipitates were split for subsequent Western blotting using anti-Flag reagents and for in vitro kinase assay.

(**Table 1**). No ions exhibiting masses indicating phosphorylated fragments were found. The analysis of IRAK-1-DDL resulted in the detection of masses as described above and additional ions displaying masses corresponding to tryptic digestion-derived fragments from the ProST region with shifts of  $n \times 80$  Da (**Table 1**). By analysis of the full-length IRAK-1 molecule by the same method, a phosphorylation of T66 could not be detected as well, and masses indicative of phosphorylated peptides out of the ProST region and the kinase domain could be identified.

### Lack of the ProST region enables stable interaction of IRAK-1 with signaling adaptors

The interaction of IRAK-1 with signaling molecules such as MyD88, Tollip, and IRAK-4 and thus, probably also the conformation of the IRAK-1 DD is regulated by phosphorylation of the IRAK-1 molecule, however not at the position T66. Upon activation, IRAK-1 is heavily phosphorylated in the ProST region adjacent to the DD [14]. Thus, it is tempting to speculate that the large number of negative charges introduced by phos-

TABLE 1. Mass Spectrometry Analysis of w.t. FL-IRAK-1-DD

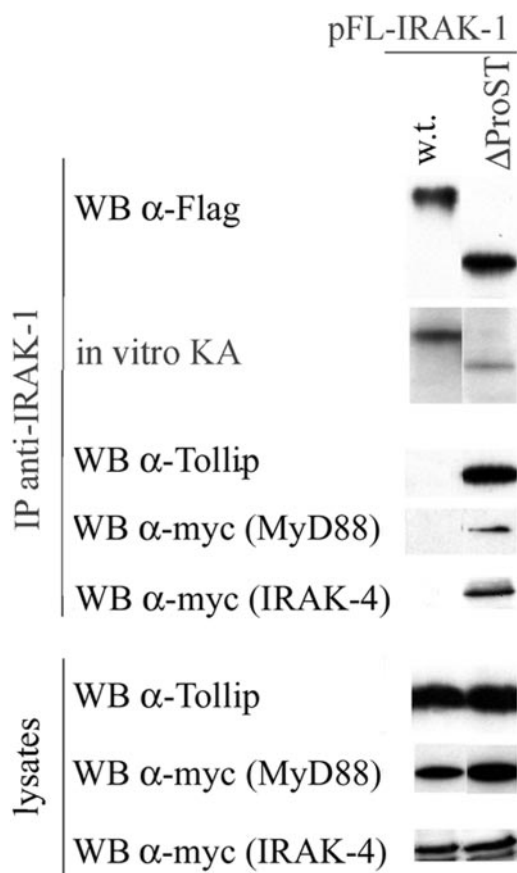
Mass	Position	# MC	Peptide sequence
<b>DDL</b>			
<b><i>4069.1041</i></b>	<b>95–133</b>	<b>1</b>	<b><i>ARDIITAWHPPAPLPSPGTTAPRPSSIPAPAEAEAWSPR</i></b>
3970.0609	97–134	1	DIITAWHPPAPLPSPGTTAPRPSSIPAPAEAEAWSPRK
3841.9659	97–133	0	DIITAWHPPAPLPSPGTTAPRPSSIPAPAEAEAWSPR
2204.0982	134–155	1	KLPSSASTFLSPAFFPGSQTHSG
2076.0032	135–155	0	LPSSASTFLSPAFFPGSQTHSG
<b>DDs and DDL</b>			
3536.5997	(–5)–29	1	DDDDKAGGPGGPEPAAPGAQHFLYEVPWVMCR
3386.6237	2–38	1	AGGPGGPEPAAPGAQHFLYEVPWVMCRFYK
2948.3970	2–29	0	AGGPGGPEPAAPGAQHFLYEVPWVMCR
2947.4440	33–57	1	VMDALEPADWCQFAALIVRDQTELRL
2643.3097	30–51	1	FYKVMdalePADWCQFAALIVR
<b>2205.0830</b>	<b>33–51</b>	<b>0</b>	<b>VMDALEPADWCQFAALIVR</b>
2082.2294	77–96	1	NARVADLVHILTHLQLLR
1968.1865	80–96	1	VADLVHILTHLQLLRAR
<b>1770.9398</b>	<b>62–76</b>	<b>1</b>	<b><u>SGQR</u>TASVLWPWINR</b>
<b>1741.0482</b>	<b>80–94</b>	<b>0</b>	<b>VADLVHILTHLQLLR</b>
1683.9077	66–79	1	<u>TASVLWPWINR</u> NAR
<b>1342.7266</b>	<b>66–76</b>	<b>0</b>	<b><u>TASVLWPWINR</u></b>
1319.6372	52–61	1	DQTELRLCER
1144.4463	(–9)–(–1)	1	MDYKDDDDK
1005.4894	58–65	1	LCERSGQR
<b>761.3788</b>	<b>52–57</b>	<b>0</b>	<b>DQTELRL</b>
607.2205	(–5)–(–1)	0	DDDDK
556.2435	(–9)–(–6)	0	MDYK
577.2762	58–61	0	LCER
<b>DDs</b>			
<b>1654.9063</b>	<b>95–109</b>	<b>1</b>	<b>ARDIITAWHPPAPLP</b>
<b>1427.7681</b>	<b>97–109</b>	<b>0</b>	<b>DIITAWHPPAPLP</b>

Mass spectrometry of Flag-tagged w.t. IRAK-1-DDS and -DDL proteins. The table shows the expected fragments and their unphosphorylated masses, arising exclusively from the IRAK-1-DDS or the IRAK-1-DDL proteins or from both of them, as indicated on the left. Ions found by mass spectrometry displaying masses that correspond to expected fragments are in bold. Ions with masses indicative of phosphorylated peptides (thus,  $+n \times 80$  of the expected mass) were found only for the 4069.0141 fragment (shown in italics). The residue T66 is shown enlarged and underlined (# MC=number of missed cleavages).

phorylation of the ProST region impacts the conformation of the adjacent DD, disabling the interactions to the signaling adaptors. To prove this assumption, we performed coimmunoprecipitation experiments followed by immunoblotting and in vitro kinase assays using an IRAK-1 deletion mutant lacking the ProST region ( $\Delta$ ProST). Lack of the ProST region did not abolish in vitro phosphorylation of the IRAK-1 molecule. Moreover, although IRAK-1  $\Delta$ ProST is phosphorylated, and its T66 is not mutated, it in contrast to full-length, w.t. IRAK-1, stably interacts with the signaling proteins Tollip, MyD88, and IRAK-4 (Fig. 6). Thus, it is the ProST region, i.e., the phosphorylations taking place within it, which regulates the interaction between IRAK-1 and the adaptor proteins Tollip, MyD88, and IRAK-4.

## DISCUSSION

The data presented here indicate that T66 of IRAK-1 is critical for the conformational structure of the DD, as indicated by the



**Fig. 6.** Deletion of the ProST region of IRAK-1 enables coimmunoprecipitation of Tollip, MyD88, and IRAK-4. Flag-tagged IRAK-1 constructs, as indicated above the figure, were expressed in HEK293RI cells alone for in vitro kinase assay or in combination with untagged Tollip, myc-tagged MyD88, or myc-tagged IRAK-4 for coimmunoprecipitation analyses. IRAK-1 proteins were immunoprecipitated with the anti-IRAK-1 antibody 2A9. Precipitates were washed and subjected to in vitro kinase assay or analyzed for coimmunoprecipitated Tollip, MyD88, and IRAK-4 by Western blotting using Tollip- and myc-specific reagents. Efficient immunoprecipitation of IRAK-1 molecules was verified by anti-Flag Western blot, and expression of Tollip, MyD88, and IRAK-4 was checked by Western blotting of whole cell lysates.

interaction of IRAK-1 with Tollip, MyD88, and IRAK-4 and by the apparent molecular weight of IRAK-1 in SDS-PAGE. Although T66 is a putative (auto-) phosphorylation site, and its phosphorylation was suggested to be the mechanism regulating the above-mentioned characteristics [6, 9], T66 is not phosphorylated by IRAK-1 itself in vitro. Moreover, it seems that upon expression in HEK293RI cells, IRAK-1 T66 is neither a target site for other kinases such as protein kinase C (PKC) $\alpha$  [10] as well. Yet it cannot be ruled out that the cells used in our study might express insufficient amounts of PKC $\alpha$  or that PKC $\alpha$ , although activated by IL-1 stimulation as well [17], has to be triggered specifically with nerve growth factor to phosphorylate IRAK-1 at T66.

In conclusion, our data suggest the following model. In quiescent cells, IRAK-1 resides inactivated and not phosphorylated. In this conformation, it interacts with Tollip. Upon TIR-mediated stimulation of the cells, IRAK-1 is recruited to the receptor complex and homo-oligomerizes. The IRAK-1 structure is not yet altered, enabling interactions with the signaling molecules MyD88 and IRAK-4 and initiation of signal transduction by binding of Traf6. Concomitantly, IRAK-1 is initially phosphorylated, most probably by IRAK-4, and thereby becomes kinase-active, autophosphorylates, alters its conformation, and dissociates from its DD-interacting molecules [14]. Finally, phosphorylated IRAK-1 is ubiquitinated and degraded at the proteasome [3].

Overexpression of kinase-inactive IRAK-1 K239S mimics the status in which IRAK-1 is homo-oligomerized but not yet phosphorylated. Therefore, this molecule is able to stably interact with the signaling adaptors to transduce TIR-generated signaling, and to induce signaling upon overexpression [12]. Overexpression of w.t. IRAK-1 mimics the homo-oligomerized and hyperphosphorylated status of IRAK-1. The interactions of this molecule with the signaling adaptors MyD88, IRAK-4, and Tollip are not stable enough to be detected by coimmunoprecipitation. It interacts with the DD-binding signaling adaptors in a transient manner, as it is able to relay on TIR-generated signaling and to induce signaling upon overexpression [12]. Traf6 could be coimmunoprecipitated with all IRAK-1 molecules as far as the C-terminal domain is present. Thus, Traf6 binds to IRAK-1 independently of the phosphorylation status of IRAK-1 and of the DD conformation (data not shown). T66 seems to have an essential role in maintaining the status, which disables interactions of IRAK-1 with the DD-binding signaling molecules. Its mutation to any other amino acid seems to mimic a status in which the conformation of the DD is altered to allow stable interactions with signaling adaptors, although the molecule is phosphorylated. Thus, it generates a status between those mimicked by the IRAK-1 K239S and the IRAK-1 w.t. molecules.

Interactions between IRAK-1 and the signaling adaptors, which are mediated by the DD [8], depend on the phosphorylation status of IRAK-1 [12] and on the conformation of the DD. However, the IRAK-1 DD itself is not phosphorylated; thus, phosphorylation of T66 cannot be the mechanism regulating the conformational alterations of the DD. Phosphorylation of IRAK-1 takes place initially within the kinase domain (T209, T387) and thereafter, at multiple residues within the ProST region and is followed by dissociation of the adaptor

molecules [14]. As IRAK-1, lacking the ProST region, still becomes phosphorylated but also stably interacts with the adaptor proteins, the phosphorylation within the ProST region must be responsible for the interactions with the adapters and therefore, also for the conformation of the DD. One might speculate that the negative charges introduced into the ProST region by phosphorylation impact the DD structure, altering the interacting structure into the not-interacting one. As full-length IRAK-1 T66 mutants, which are phosphorylated, stably interact with the adaptor proteins, it might be speculated that the impact of the phosphorylated ProST region on the DD conformation is mediated by T66. The biophysical nature of this function is still to be determined.

It remains to be elucidated whether in chronic inflammatory diseases such as rheumatoid arthritis modifications within the IRAK-1 DD, which lead to stable interactions of the signaling molecules, could contribute to chronicity, e.g., by the enhanced, spontaneous production of proinflammatory cytokines. In such case, the modification of the half-life of the signaling complex would serve as a promising therapeutic target.

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