Identification of Threonine 66 as a Functionally Critical Residue of the Interleukin-1 Receptor-associated Kinase*

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We have mutated a conserved residue of the death domain of the interleukin-1 (IL-1) receptor-associated kinase (IRAK), threonine 66. The substitution of Thr-66 with alanine or glutamate prevented spontaneous activation of NF-κB by overexpressed IRAK but enhanced IL-1-induced activation of the factor. Like the kinase-inactivating mutation, K239S, the T66A and T66E mutations interfered with the ability of IRAK to autophosphorylate and facilitated the interactions of IRAK with TRAF6 and with the IL-1 receptor accessory protein, AcP. Wild-type IRAK constructs tagged with fluorescent proteins formed complexes that adopted a punctate distribution in the cytoplasm. The Thr-66 mutations prevented the formation of these complexes. Measurements of fluorescence resonance energy transfer among fluorescent constructs showed that the Thr-66 mutations abolished the capacity of IRAK to dimerize. In contrast, the K239S mutation did not inhibit dimerization of IRAK as evidenced by fluorescence resonance energy transfer measurements, even though microscopy showed that it prevented the formation of punctate complexes. Our results show that Thr-66 plays a crucial role in the ability of IRAK to form homodimers and that its kinase activity regulates its ability to form high molecular weight complexes. These properties in turn determine key aspects of the signaling function of IRAK.

All of the biological effects of interleukin 1 (IL-1) depend upon its binding to the type I IL-1 receptor, IL-1RI (1). The receptor is the prototypic member of a diverse family of conserved proteins generally involved in host defense (2). They include the Drosophila Toll protein (3), the mammalian Toll-like receptors, the IL-18 receptor and components of IL-1 signal transduction pathways such as the IL-1RI accessory protein (AcP), and the intracellular adapter protein MyD88 (2, 4). The family is characterized by a conserved intracellular motif (the Toll/interleukin-1 receptor domain), suggesting that all members couple to similar signaling pathways (2).

The binding of IL-1 to IL-1RI initiates the formation of a complex that includes IL-1RI, AcP, the adapter protein MyD88, and the IL-1RI-associated protein kinases (IRAKs). They consist of IRAK, IRAK-2, IRAK-4, and in cells where it is expressed, IRAK-M (4–9). Subsequent events involve the phosphorylation of IRAK by itself and by IRAK-4, its dissociation from the IL-1R1 complex, its ubiquitination, and its association with two membrane-bound proteins, the TGF-β-activated kinase (TAK1)-binding protein 2 (TAB2) and TRAF6, a member of the tumor necrosis factor (TNF) receptor-associated factor (TRAF) family of adapter proteins (8, 10–13). The resulting IRAK-TRAF6-TAB2 (TAK1-binding protein 2) complex is then released into the cytoplasm and activates protein kinase cascades, which include TAK1, the IκB kinases, and the stress-activated protein kinases (13–18). The roles of the two IRAK homologues, IRAK-2 and IRAK-M, are less well understood than the roles of IRAK and IRAK-4. Like IRAK, they associate with the IL-1R1 complex (8, 19). They have been shown to reconstitute the IL-1 response in a cell line lacking IRAK (8, 19).

IRAK undergoes autophosphorylation shortly after stimulation of cells by IL-1, indicating that its catalytic activity is modulated during IL-1 signal transduction (11). However, a point mutation of lysine 239 that completely abolished its catalytic activity did not interfere with its ability to activate NF-κB when expressed at high levels in transfected cells, suggesting that the protein kinase activity may not play an important role in signaling (4, 9, 19–23).

The four human IRAKs and their Drosophila homologue, Pelle, all contain an NH₂-terminal death domain. The death domain is a protein interaction domain, first defined as a conserved motif in the cytoplasmic domains of Fas and the type I TNF receptor (24), and has since been found in proteins of diverse cellular functions including MyD88 (25, 26). The death domains of IRAKs mediate their recruitment to the IL-1RI complex by associating with the death domain of MyD88 (4, 8). They may also be involved in associations between IRAK molecules. This may be the reason why deletion of its death domain prevents overexpressed IRAK from spontaneously autophosphorylating, associating with TRAF6, and activating NF-κB (13, 17, 23).

We have investigated the functional roles of two conserved residues in the death domain of IRAK, Thr-66 and Trp-73. We have found that threonine 66 played a crucial role in the ability of IRAK to form homo-oligomeric complexes, a property that appears to regulate many aspects of IRAK function. Our results...
support a model in which the signaling activity of IRAK is regulated by both autophosphorylation and self-assembly.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**Cells lines (COS-1 and NIH 3T3) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected using Superfect (Qiagen).

**Eukaryotic Expression Constructs—**The FLAG-tagged TRAF6 and Adp cDNA constructs, the IRAK expression construct, and the NF-κB-responsive luciferase reporter plasmid (pNF-κB) have been described elsewhere (27, 28). IRAK mutants were generated by site-directed mutagenesis of the IRAK expression construct with the QuikChange kit (Stratagene).

**EGFP-IRAK, YFP-IRAK, and CFP-IRAK Constructs—**The wild-type IRAK sequence was excised from a pBluescript construct (28) with Sali and SceI restriction sites, and ligated into the same sites of the expression vectors pEGFP-C1, pEYFP-C1, or pECFP-C1 (CLONTECH) downstream of sequences encoding the enhanced green (EGFP), yellow (YFP), or cyan (CFP) variants of *Aequorea victoria* green fluorescent protein, respectively. The mutant IRAK cDNAs were transferred into pBluescript using EcoRI and BamHI restriction sites and then excised from cDNA constructs, ligated into SphI and Sall restriction sites and ligated into pEGFP-C1 or pEYFP-C1 similar to wild-type IRAK. Sequencing of the constructs confirmed that they encoded in-frame fusions of wild-type or mutant IRAK to the carboxyl termini of EGFP, YFP, or CFP.

**GFP-IRAK-2 and CFP-IRAK-2 Constructs—**The full-length IRAK-2 coding sequence was amplified using primers of sequences 5′-GAATTCACACTCCCACAGGAAAAATTTGGAGGAACAGCAGG-3′ and 5′-GGAATTCGGCGCACACGCTGCTGTTCGACCTGC-3′. This construct encodes a fusion protein consisting of YFP linked to the IRAK death domain.

**CFP-YFP Construct—**This construct encodes a fusion protein consisting of CFP joined to carboxyl end of CFP by a 15- residue linker of sequence GGRRQAADPDVAT. It was constructed by Dr. B. Eckenrode.

**Reporter Assays—**COS-1 cells were seeded at 3 × 10⁵ cells/well in six-well plates and transfected the next day with 1 μg of pNF-κB, 0.2 μg of pCMV-β-galactosidase (27), and varying amounts of expression constructs. Total DNA was kept constant by the addition of empty vector. The cells were harvested and lysed 2 days later (7). Luciferase and β-galactosidase activities were assayed as described previously (27). Luciferase readings were normalized to β-galactosidase values.

**Confocal laser scanning microscopy of EGFP- and YFP-expressing Cells—**COS-1 cells were seeded (5 × 10⁴/well) in four-well chambered coverglasses (NalgeNunc) and transfected the following day with 2 μg of plasmid. Images were acquired with a Molecular Dynamics confocal microscope equipped with a krypton/argon ion laser and controlled by a Silicon Graphics work station. Fluorescence excitation was performed with a 488-nm line of the laser. Fluorescence was collected through a 530 ± 15-nm bandpass filter. Images were obtained with an ×100 Plan Apo Immersion lens fitted on a Nikon Diaphot microscope.

**Detection of Fluorescence Resonance Energy Transfer (FRET)—**NIH 3T3 cells were seeded in chambered coverglasses as above and transfected the next day with expression plasmids for CFP- and YFP-labeled proteins (0.5 μg each). Images were recorded 24 h later with a Nikon Diaphot 300 microscope equipped with a mercury arc lamp and a 10-bit digital CCD camera (Hamamatsu). Control of the camera and quantitative analysis of images were performed with OpenLab software (Image Processing and Vision Co. Ltd.). Images of CFP and YFP fluorescence were acquired using the XP114 (excitation 440 nm, emission = 480 nm) and YFP (excitation 505 nm emission 535 nm) filter sets, respectively (Omega Optical, Brattleboro, VT). FRET was detected with a custom-made filter set (excitation 440 nm, emission 535 nm), also from Omega Optical.

Emission intensities were measured on the cytoplasms of individual cells. Each measurement was corrected by subtraction of background fluorescence determined from an extracellular region of the same image. FRET measurements were then corrected by subtraction of the direct emissions from donor and acceptor. These were calculated from the CFP and YFP measurements using correction factors determined in calibration tests done on cells expressing CFP and YFP (individually and in co-transfections) as well as the CFP–YFP construct. The FRET intensity measurements were normalized to CFP fluorescence (30).

**Immunoprecipitation and Immunoblotting—**COS-1 cells were seeded at 3 × 10⁵ cells/10-cm dish and transfected on the following day. At 24 h post-transfection, cells were lysed and immunoprecipitations were performed as described previously (28, 29) with 4.4 μg of anti-FLAG M2 antibody (Sigma) and protein G-Sepharose (Amersham Pharmacia Biotech). The beads were then washed three times with lysis buffer without glycerol, resuspended in 50 μl of 2× SDS-PAGE sample buffer, and heated at 100 °C for 1 min. A 20-μl aliquot was separated on SDS Tris-HCl gels containing a gradient of 4–20% polyacrylamide (Bio-Rad) and transferred to polyvinylidene difluoride membranes. The membranes were probed with anti-IRAK (28) at 1:5000 dilution or anti-FLAG antibodies (1:2000 dilution) and in co-transfections) as well as the CFP–YFP construct. The FRET intensity measurements were normalized to CFP fluorescence (30).

**RESULTS**

**Identification of Potentially Important Residues of the IRAK Death Domain—**Death domains consist of six α-helices packed into a cylindrical structure (31, 32). By analyzing the sequences of several death domains and the crystal structures of the death domains of the *Drosophila* proteins Pelle and Tube (32), we identified two conserved residues that may play important roles in IRAK function (Fig. 1).

The most conserved residue is a threonine or serine that is specifically conserved in the IRAK family and in several other proteins such as Tube, MyD88, and the NF-κB family members, p100 and p105. It is located at the amino-terminal end of the fourth helix and corresponds to Thr-66 of IRAK, Ser-83 of Pelle, or Ser-110 of Tube (Fig. 1). An analysis of the crystallographic data with RasMol (34) shows that Ser-83 and Ser-110 are not exposed at the surface of the Pelle and Tube death domains. Instead, they are hydrogen-bonded to a conserved residue of the first α-helix, Asp-50 of Pelle and Asp-49 of Tube. This finding strongly suggests that the homologous residue of IRAK, Asp-35, is hydrogen-bonded to Thr-66 and that this links to gather the IRAK expression and first helices.

In the case of the locations of Thr-73 and Thr-66 at opposite ends of the fourth α-helix, we reasoned that mutating each residue would enable us to destabilize independently the packing of α-helices at either end of the death domain of IRAK and produce qualitatively distinct structural effects. In particular, replacing Thr-66 by a negatively charged residue should have a strong effect by creating electrostatic repulsion between Gln-66 and the positively charged Thr-73.
Differential Effects of Mutations of IRAK on NF-κB Activation—The activation of NF-κB is a key component of cellular responses to IL-1. Thus, we monitored the effects of the mutations on the NF-κB-stimulating activity of IRAK in cells transfected with the promoter/reporter construct, pNF-κBBluc (Fig. 2A). As previously reported (19), wild-type IRAK stimulated NF-κB in a dose-dependent manner in the absence of IL-1. Cells transfected with 0.5 μg of an IRAK expression construct produced 20 times more luciferase than control cells (Fig. 2A). In agreement with published results (4, 9, 19–23), we also found that the kinase-suppressing mutation, K239S, did not interfere with this effect of IRAK (data not shown). In contrast, both of the T66A and W73A mutations strongly reduced the ability of IRAK to spontaneously activate NF-κB. In cells transfected with 0.5 μg of each mutant construct, the increase in reporter expression relative to controls was 5-fold (Fig. 2A). Immunoblotting of the cell lysates with the anti-IRAK antibody showed that the fluorescent moiety interfered with the activity of IRAK, as indicated by the reduced amount of the corresponding unlabeled proteins to activate NF-κB. The level of activation induced by the fluorescent and non-fluorescent versions was not significantly different, indicating that fusion of the amino-terminal end of IRAK to a fluorescent protein did not alter its signaling function (data not shown).

Confocal fluorescence microscopy of live COS-1 cells expressing low amounts of EGFP-IRAK showed that it adopted a punctate distribution throughout the cytoplasm and was excluded from the nucleus (Fig. 3A). This indicated that most or all of the fluorescent IRAK existed in discrete high molecular weight complexes. Higher levels of expression resulted in the accumulation of IRAK particles in very large patches (Fig. 3B). Low intensity imaging showed the patches to be composed of multiple copies of the complexes observed at low expression levels (data not shown).

An analysis of high resolution images of cells expressing EGFP-IRAK with the Sobel edge detection algorithm showed that the fluorescent particles had an elongated shape (Fig. 3C). Their widths were smaller than the optical resolution (0.2 μM), whereas their lengths appeared to vary between 0.2 and 1 μM (Fig. 3C). This variation may result from random orientation of the particles relative to the image plane, rather than from true heterogeneity. A quantitative analysis of the fluorescence intensities of the particles in cells covering a 10-fold range in expression level showed that the average number of fluorescent molecules that they contained increased with expression once a threshold had been reached, suggesting that there is a concentration-driven assembly process within the cell (Fig. 3D).

The YFP-IRAK-(W73A) fusion protein adopted the same punctate distribution as EGFP-IRAK (Fig. 4A). In contrast, YFP-IRAK-(T66A), YFP-IRAK-(T66E), and YFP-IRAK-(K239S) were all uniformly distributed throughout the cytoplasm (Fig. 4, B–D). This indicated that the formation of the high molecular weight IRAK complexes required both the presence of Thr-66 in the death domain and the catalytic activity of IRAK.
We then examined the ability of mutant IRAK proteins to self-associate in live transfected cells by measuring FRET between pairs of wild-type or mutant IRAK proteins labeled with CFP and YFP. The technique provides a sensitive way of detecting direct interactions between fluorescent fusion proteins (30).

Co-expression of CFP-IRAK and YFP-IRAK constructs resulted in a FRET signal, indicating that the technique could detect the self-association of IRAK. The signal was significantly reduced in control experiments in which a non-fluorescent IRAK protein was co-expressed with the CFP- and YFP-tagged versions (data not shown). This finding confirmed that the FRET signal was the result of specific and saturable interactions. Co-expression of the YFP- and CFP-tagged versions of the K239S mutant also generated a FRET signal, indicating that the kinase-inactive mutant was capable of self-association (Fig. 5, A and C). In contrast, the T66A and T66E mutations completely abolished the signal (Fig. 5, B and C). Quantitative analysis of the FRET images also showed that the K239S mutant constructs produced a FRET signal whose intensity was similar to those obtained with wild-type IRAK, the W73A mutant, or the CFP-YFP fusion protein that we used as a positive control (Fig. 5, C).

These results establish Thr-66 as a critical determinant of the ability of IRAK to dimerize. They also show that failure of the kinase-inactive mutant to form punctate complexes did not result from an impaired ability to dimerize.
The average FRET intensities measured for the YFP-IRAK-M was used instead of CFP-IRAK-2 (data not shown). Similar results were obtained when CFP-tagged versions of IRAK-2 or IRAK-M with the YFP-IRAK constructs.

When co-transfected with wild-type YFP-IRAK, CFP-IRAK-2 was uniformly distributed in the cytoplasm and the nucleus (Fig. 6, A and E). The same distribution was observed for CFP-IRAK-M (data not shown). In these experiments, YFP-IRAK adopted the same punctate distribution than when over-expressed alone (compare Figs. 6B and 3B). This was evidence that the majority of the YFP-IRAK and CFP-IRAK-2 pools were not interacting. A FRET signal whose distribution was identical to that of the YFP-IRAK complexes was also detected (Fig. 6, C and D), indicating that a fraction of the CFP-IRAK-2 was physically bound to the YFP-IRAK complexes. The FRET signal would have instead been distributed evenly if interaction with CFP-IRAK-2 had prevented the recruitment of YFP-IRAK into the complexes. Similar results were obtained when CFP-IRAK-M was used instead of CFP-IRAK-2 (data not shown). The average FRET intensities measured for the YFP-IRAK-IRAK2 and YFP-IRAK-CFP-IRAK-M pairs (Fig. 7) were significantly weaker than the values obtained in the case of the YFP-IRAK-CFP-IRAK interaction (Fig. 5). These results show that only a small fraction of the CFP-IRAK-2 or CFP-IRAK-M pools associated with YFP-IRAK and that the associations did not interfere with the assembly of IRAK into punctate complexes.

The mutations that prevented IRAK from adopting a punctate distribution (K239S, T66A, and T66E) resulted in FRET being observed throughout the cytoplasm (Fig. 6, E–H). However, measurements showed that substitution of Thr-66 of IRAK with either alanine or glutamate had no significant effect on the FRET intensities (Fig. 7). Thus, the dramatic changes in both the intracellular distribution and quaternary structure of IRAK brought about by mutations of Thr-66 had no impact on the ability of IRAK to associate with its two cognates. In contrast to the Thr-66 mutations, the K239S mutation increased the FRET signal measured between YFP-IRAK and either CFP-IRAK2 or CFP-IRAK-M by more than 2-fold (Fig. 7). The effect was highly significant ($p < 10^{-4}$) in both cases. This result indicated that the kinase activity of IRAK was reducing its ability to associate with IRAK2 and IRAK-M.

Interactions of IRAK with IRAK-2 or IRAK-M—Because IRAK can heterodimerize with IRAK-2 and IRAK-M, we then asked whether these heterologous interactions would prevent the formation of the IRAK high molecular weight complexes and whether the mutations of IRAK would interfere with its associations with IRAK-2 and IRAK-M. Thus, we co-transfected CFP-tagged versions of IRAK-2 or IRAK-M with the YFP-IRAK constructs.

To determine the effects of the death domain mutations on coupling of IRAK to the immediate upstream and downstream steps in the signaling pathway, we then examined the effects of the mutations on the association of IRAK with FLAG epitope-tagged versions of AcP and TRAF6. Previous studies have shown that IRAK-(K239S) binds to both proteins, whereas overexpressed wild-type IRAK does not (4). In agreement with these findings, we observed that IRAK-(K239S) but not wild-type IRAK was present in the immunoprecipitates of the FLAG-AcP protein (Fig. 8A). The W73A mutant behaved exactly as the wild-type IRAK, failing to associate with FLAG-tagged AcP (Fig. 8A). The IRAK-(W73A,K239S) double mutant showed that as in the case of wild-type IRAK, suppression of the kinase activity of IRAK-(W73A) allowed it to interact with FLAG-AcP. Thus, the W73A mutation did not affect the latent ability of IRAK to associate with AcP.
Both of the IRAK-(T66A) and IRAK-(T66E) mutants were present in much larger amounts than IRAK-(K239S) and IRAK-(W73A,K239S) in the FLAG-AcP immunoprecipitates (Fig. 8A). Control immunostaining of the membrane with a FLAG-specific antibody showed that the immunoprecipitates contained similar amounts of FLAG-AcP protein (data not shown), and direct immunoblots of the cell lysates with the antibody to IRAK showed that the wild-type and mutant versions of IRAK were expressed at similar levels (Fig. 8B).

Almost identical results were obtained when the interactions of fluorescent IRAK constructs with FLAG-tagged TRAF6 were tested (Fig. 8C). No interaction of FLAG-TRAF6 with either CFP-IRAK or CFP-IRAK-(W73A,A239S) could be detected, whereas CFP-IRAK-(K239S), YFP-IRAK-(T66A), and YFP-IRAK-(T66E) were all detected in the FLAG-TRAF6 immunoprecipitates (Fig. 8C). Like the K239S mutant, the double mutant IRAK-(W73A,K239S) was found in the FLAG-TRAF6 immunoprecipitates (data not shown). Control immunoblots confirmed that the fluorescent IRAK constructs (Fig. 8D) and FLAG-TRAF6 (data not shown) were expressed at similar levels in all samples.

Autophosphorylation of IRAK has been shown in numerous reports to reduce its electrophoretic mobility on polyacrylamide gels (4, 11, 19, 23). We investigated the phosphorylation state of the mutant and wild-type IRAK proteins by Western blotting of lysates of transfected COS-1 cells. In agreement with published results (4, 11, 19, 23), we found that wild-type IRAK was retarded on SDS-PAGE gels relative to the kinase-inactive mutant (Fig. 8D). The W73A mutant had the same mobility as wild-type IRAK. In most experiments, the IRAK-(T66E) and IRAK-(T66A) mutants migrated as single bands close to the position of IRAK-(K239S), indicating that the substitutions of Thr-66 prevented its autophosphorylation (Fig. 8D). However, in some experiments, a fraction of the IRAK-(T66A) population migrated at a position intermediate between those of wild-type and kinase-dead IRAK, indicating that a fraction of the IRAK-(T66A) pool was partially phosphorylated (Fig. 8D). Thus, although both substitutions of Thr-66 impaired the ability of IRAK to autophosphorylate, the substitution to alanine had a less severe effect than the substitution to glutamate.

**DISCUSSION**

We found at the beginning of this study that GFP-IRAK accumulated into complexes that adopted a punctate distribution and resulted from a concentration-driven assembly process. While this work was in progress, endogenous IRAK was shown by others (35) to distribute in punctate complexes in both unstimulated and IL-1-stimulated ECV 304 cells. We have also made a similar observation in unstimulated mouse 3T3 fibroblasts.2 These results indicate that endogenous IRAK exists as multimeric complexes both before and after activation. The observation that IRAK eluted from a gel filtration column at a molecular size consistent with a homodimer (17) indicates that dimers are the stable form of the protein in diluted extracts and that they probably constitute the basic building block of the complexes.

The fluorescent wild-type IRAK complexes observed in this study contained fully phosphorylated IRAK, and they activated NF-κB spontaneously. Thus, they were functionally similar to the endogenous IRAK particles observed after IL-1 stimulation (35), which could correspond, at least in part, to IRAK-TRAF6/TAK1 signaling complexes. By contrast, the endogenous IRAK complexes present in unstimulated cells (35) do not activate NF-κB, presumably because they cannot recruit downstream signaling components such as TRAF6.

To dissect the structural basis of these effects, we mutated two conserved residues of the death domain, Thr-66 and Thr-73, and compared their effects to that of the kinase-inactivating mutation. K239S. The only effect of the W73A mutation was to reduce the spontaneous activation of NF-κB by overexpressed IRAK. Thus, Thr-73 is not a functionally critical residue. This was surprising given that the tryptophan is conserved in all death domains (25, 26, 32) and that its homologue in the type I TNF receptor is essential for cytotoxic signaling (33). This discrepancy suggests that protein-protein interactions involving the death domain of IRAK could be mechanistically different from those of other death domains such as that of the type I TNF receptor.

In contrast, the Thr-66 substitutions affected every aspect of IRAK activity that we examined with the exception of its interactions with IRAK-2 or IRAK-M. The mutations prevented spontaneous activation of NF-κB while amplifying its activation by IL-1. They also abolished the ability of IRAK to self-associate, prevented its recruitment into punctate complexes, and facilitated its associations with AcP and TRAF6. These effects identify Thr-66 as a functionally crucial residue of the death domain of IRAK.

We have shown in a separate report that the T66A mutation stabilizes the association of IRAK with another pathway com-

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2 F. Volpe, unpublished results.
ponent, Tollip (29). Tollip plays an inhibitory role in both IL-1 and Toll-like receptor function by promoting the ubiquitination and degradation of IRAK (29, 36). However, degradation mediated by endogenous Tollip cannot account for the effects of the Thr-66 mutants in this study given the high expression levels of the proteins detected on the immunoblots.

The electrophoretic mobility of the Thr-66 mutants indicated that the mutations also affected the phosphorylation state of IRAK. IR-AK-(T66A) appeared to be partially phosphorylated in some experiments, whereas the T66E mutation completely abolished phosphorylation. Thus, the ability of IRAK to autophosphorylate depended on the structural integrity of the death domain in the region spatially adjacent to Thr-66. The more dramatic effect of the T66E mutation compared with T66A is in agreement with our prediction that the former mutation would cause the stronger structural alterations via electrostatic repulsion between Glu-66 and Asp-35. Two mechanisms may account for the impaired autophosphorylation of the Thr-66 mutants. First, their inability to self-associate probably inhibited strongly the intermolecular autophosphorylation reactions. Second, the structural disruptions caused by the mutations may have prevented the recognition of certain sites by the catalytic domain.

Similar to the Thr-66 substitutions, the K239S mutation favored the association of IRAK with AcP and TRAF6. It also facilitated the interactions with IRAK-2 and IRAK-M. These results are in agreement with earlier reports showing that IRAK-(K239S) co-precipitated with IRAK-2, IRAK-M, MyD88, or TRAF6 more efficiently than wild-type IRAK (4, 9, 19–23). In addition, we found that the K239S mutation prevented assembly of IRAK into punctate complexes, but unlike the Thr-66 mutations, it did not impair its ability to self-associate.

In view of the gel filtration results of Cooke et al. (17), it appears very probable that the FRET signal was produced by a dimeric form of the fluorescent IRAK-(K239S) constructs. The inability of the IRAK-(K239S) dimers to form high molecular weight complexes indicates that their formation requires autophosphorylation of IRAK. Overall, the K239S mutation had a less profound effect on IRAK function than the Thr-66 mutations, because it did not affect the ability of IRAK to spontaneously activate NF-κB when overexpressed (20–23).

The interactions of AcP or TRAF6 with IRAK-(K239S) have been interpreted before as evidence that the autophosphorylation of IRAK had an inhibitory function by preventing its interactions with other pathway components (4). Our finding that the mutation also prevented the formation of IRAK complexes suggests instead that it was facilitating the association with TRAF6 and the IL-1R1 complex indirectly by allowing IRAK to diffuse in the cytoplasm. The same mechanism may explain the association of AcP and TRAF6 with the Thr-66 mutants.

One of the most interesting discoveries in our findings is probably the enhanced IL-1-dependent activation of NF-κB caused by the Thr-66 mutants. This activation differentiates them dramatically from IRAK-(K239S), which activates the transcription factor spontaneously but is not IL-1-responsive. Our results suggest that failure of the Thr-66 mutants to spontaneously activate NF-κB may result from their inability to self-associate, which could lead to the sequestration of endogenous pathway components in an inactive state. By contrast, the association of IRAK-(K239S) dimers with endogenous components may lead to the activation of the latter by bringing them in close contact. For example, IRAK-(K239S) could stimulate endogenous TRAF6 by promoting its dimerization, which is sufficient to activate NF-κB (13, 37).

The Thr-66 mutations also facilitated the recruitment of IRAK to the IL-1R1 complex as evidenced by interactions with AcP. This may have allowed the Thr-66 mutants to participate in the IL-1 signaling pathway by recruiting downstream endogenous components to the receptor complexes. Thus, the structural effects of the Thr-66 mutants can potentially account for the ability of the mutants to enhance IL-1-induced NF-κB activation. Identifying the endogenous proteins that associate with the mutants will need to be tested this model and may also help unraveling the mechanism of action of endogenous wild-type IRAK.

In conclusion, our results provide strong evidence that the signaling activity of IRAK is regulated directly by its ability to self-associate in either diffusible homodimers or discrete high molecular weight complexes. We have found that a residue of the death domain, Thr-66, is a critical determinant of the ability of IRAK to dimerize, that the formation of IRAK complexes requires its autophosphorylation, and that the diffusible forms of IRAK (dimeric or monomeric) are more readily available for interaction with the IL-1R1 complex and TRAF6 than the high molecular weight form.

This suggest a model of IRAK function in which the complexes of endogenous IRAK present in unstimulated cells may be a form of storage of the inactive protein. Following activation by Toll/Interleukin-1 receptor domain-containing receptors, the active phosphorylated IRAK then reassembles into cytoplasmic complexes capable of recruiting and activating downstream pathway components. This phosphorylation-dependent assembly may have two possible functions. It may either increase the efficiency of the pathway or regulate the rate of proteolysis of the activated IRAK. Both hypotheses are consistent with the apparent lack of effect of kinase-suppressing mutations in transfection experiments where IRAK is overexpressed.

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