Supporting Information

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SI Materials and Methods

Protein Expression and Purification. The construct of the WT human IRAK1 kinase domain (residues 194 to 530) was PCR cloned into the pFastBac HTb vector (Invitrogen) containing an N-terminal His6 tag with a tobacco etch virus (TEV) protease cleavage site. The baculovirus was generated and amplified by two rounds in sf9 monolayer insect cells. Harvested baculovirus was used to infect suspension sf9 cells for 48 h. Harvested cells were resuspended in lysis buffer containing 25 mM Hepes at pH 7.0, 500 mM NaCl, 10 mM imidazole, 10% glycerol, and 1 mM Tris(2-carboxyethyl)phosphine (TCEP), lysed by sonication, and then centrifuged at 125,000 × *g* for 1 h. The supernatant was incubated with HisPur Cobalt Resin for 1 h and then poured into a column. After extensive washing using the lysis buffer, the bound protein was eluted with 150 mM imidazole in the lysis buffer. The eluted IRAK1 fractions were incubated with the TEV protease at 4 °C overnight to cleave the N-terminal His6 tag, and the TEV cleavage was performed under dialysis to remove imidazole. The mixture was reloaded onto a HisPur Cobalt column to remove the cleaved His6 tag and the TEV protease that was also His6-tagged. The protein was further purified by size exclusion chromatography (SEC) in the gel-filtration buffer of 20 mM Hepes at pH 7.0, 500 mM NaCl, and 1 mM TCEP, and concentrated to 5 mg/mL. Recombinant IRAK4 was prepared as previously described (10).

For reconstitution of the MyD88/IRAK4/IRAK1 Myddosome, the PCR fragment of human IRAK1 (residues 1 to 524) was inserted into the pFastBac1 vector containing an engineered N-terminal His-MBP tag with a 3C protease cleavage site while the human MyD88 death domain (residues 20 to 117) with N-terminal His tag and full-length human IRAK4 were cloned into the pFastBacDual vector. Generation of Bacmid was performed as detailed above. Harvested baculovirus containing His-MyD88 and IRAK4 and baculovirus containing His-MBP-IRAK1 were used to coinfect suspension sf9 cells at a 1:1 ratio for 48 h. Harvested cells were resuspended in lysis buffer containing 20 mM Hepes at pH 7.5, 200 mM NaCl, 20 mM imidazole, and 1 mM TCEP, lysed by sonication, and then centrifuged at 125,000 × *g* for 1 h. The affinity purification procedure with Ni-NTA resin followed the manufacturer's instructions. The eluted fraction from Ni-NTA was loaded onto a Hitrap Q (GE Healthcare) column for ion exchange purification. The high-salt fraction was collected and concentrated for gel-filtration chromatography on a Superdex 200 column, with the buffer condition of 20 mM Hepes at pH 7.5, 200 mM NaCl, and 1 mM TCEP.

Thermal Shift Assays. For the salt concentration screen, 2.5 μM IRAK1 kinase domain protein was diluted into the different buffers and mixed with Protein Thermal Shift Dye (Life Technologies) to a 20-μL volume in a 96-well plate. For the JH-I-25 binding assay, 2.5 μM IRAK1 kinase domain protein was preincubated with 100 μM JH-I-25 on ice for 10 min in 20 mM Hepes at pH 7.5 and 150 mM NaCl. Thermal scanning (25 °C to 75 °C at 1.5 °C/min) was performed using a real-time PCR setup (StepOnePlus; Applied Biosystems).

Kinase Assays. Kinase activity was determined using the ADP-Glo kinase assay (Promega) according to the manufacturer's protocol. Generally, a WT or kinase dead mutant protein was incubated with 200 μM ATP and 0.1 μg/μL myelin basic protein at room temperature for 60 min in an assay buffer containing 20 mM Hepes at pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 50 mM NaF, and 0.01% Brij-35. An equal volume of ADP-Glo reagent was added to terminate the kinase reaction and deplete the remaining ATP. ADP was then converted to ATP, which was measured by luminescence.

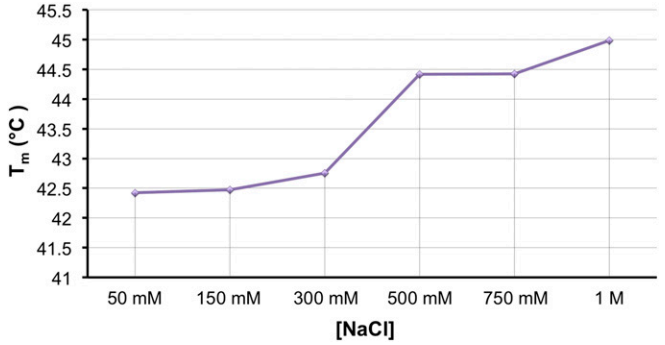

Fig. S1. Stability of recombinant IRAK1 in different salt concentrations as measured by differential scanning fluorimetry (DSF). T_m, melting temperature.

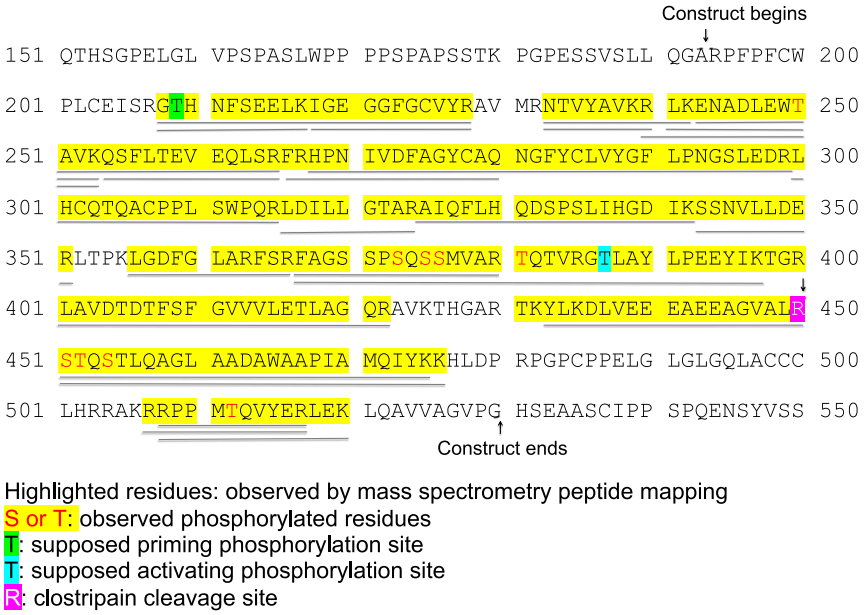

Fig. S2. Phosphorylation site mapping of natively phosphorylated IRAK1 by mass spectrometry. Observed individual peptides are marked below the sequence.

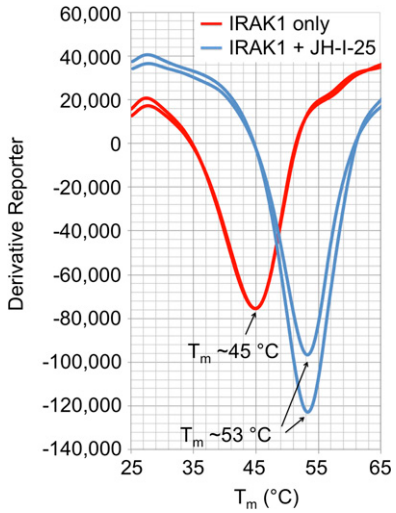

Fig. S3. Shift in IRAK1 melting temperature upon binding of JH-I-25, as measured by differential scanning fluorimetry (DSF). Two measurements are shown for IRAK1 alone and IRAK1 with JH-I-25, respectively.

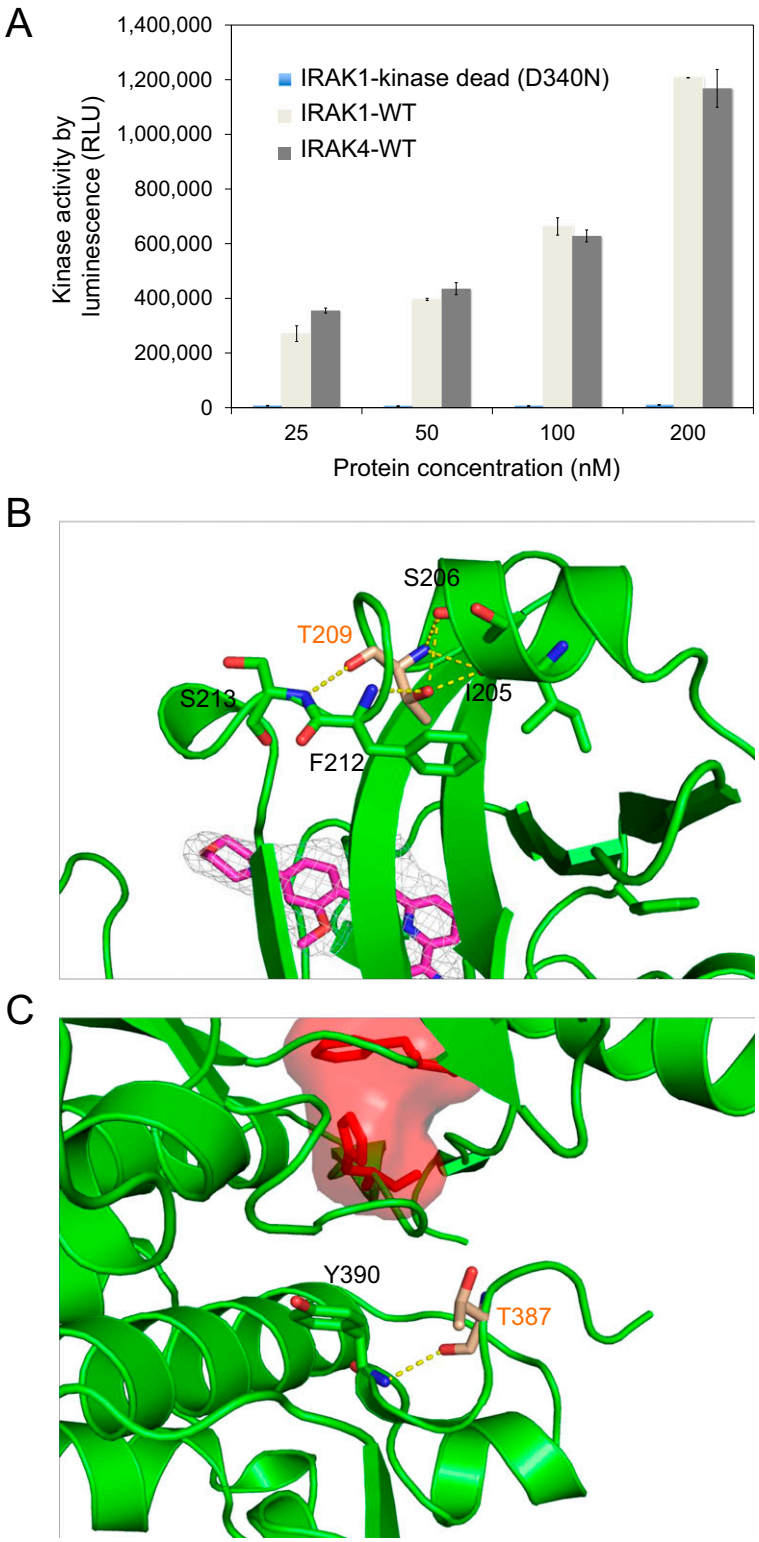

Fig. S4. The structural environments of two threonine residues that have been proposed to be phosphorylated and important for IRAK1 activation. (*A*) Catalytic activity of the IRAK1 kinase domain in comparison with the IRAK4 kinase domain. IRAK1 kinase-dead mutant was used as negative control. (*B*) Interactions by the T209 side chain, suggesting that its phosphorylation may induce structural changes. (*C*) Lack of interactions by T387, suggesting that its phosphorylation may not cause substantial conformational changes.

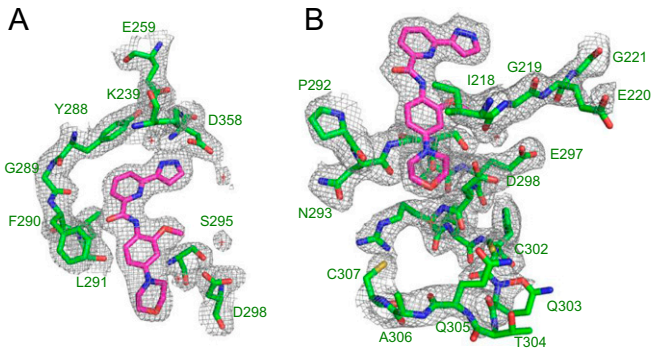

Fig. S5. Shown are 2Fo−Fc electron density maps of the active site. (*A*) Electron density at the inhibitor-binding site, shown at σ of 1.0. (*B*) Electron density at the ATP front pocket, shown at σ of 1.0.

Table S1. Crystallographic statistics

Construct	Human IRAK1 (R194–G530)
Data collection	
Space group	$P4_12_12$
Cell dimensions	
a, b, c, Å	88.1, 88.1, 177.0
α, β, γ, °	90.0, 90.0, 90.0
Wavelength, Å	0.9792
Resolution, Å	78.84–2.26 (2.33–2.26)
Total reflections	414,885 (38,428)
Unique reflections	33,471 (3,010)
Completeness, %	100.0 (100.0)
I/σ	13.3 (2.1)
$R_{merge,}$ %	14.6 (140)
Multiplicity	12.4 (12.8)
Refinement	
Molecules/au	2
$R_{work}/R_{free,}$ %	18.4 (33.4)/22.7 (34.9)
rms deviations	
Bond length, Å	0.005
Bond angles, °	0.92
Ramachandran plot, %	
Most favored regions	98
Outliers	0
Average B-factors, $Å^2$	52.2
Protein	52.5
Ligand	38.4
Solvent	47
Clash score	2.64 (100th percentile)
Molprobity score	1.54 (99th percentile)

Numbers in parentheses represent the highest resolution shell. au, asymmetric unit.