Supporting Information

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SI Materials and Methods

Plasmids. The double-tagged pPacPL FRH-Relish with FLAG and RGSH6 epitopes for cellular expression and pCITE-2a(+) FLAG-Relish for in vitro translation were previously described (1). Point mutations were generated by PCR-based site-directed mutagenesis. Mutant Relish constructs were cloned into pCITE-2a(+) and pPacPL by using standard methods. V5-tagged Dredd was cloned into pMT/V5-His expression vector (Invitrogen) controlled by the metallothionein promoter.

Cell Culture. Drosophila S2* cells were grown in Schneider's medium (Gibco) with 10% fetal bovine serum, 1% Glut-MAX (Gibco), and 0.2% penicillin–streptomycin (Gibco) at 27 °C.

dsRNA, DNA Transfections, and Stable Cell Lines. dsRNA was produced by using T7 RiboMAX Express RNAi System (Promega). S2* cells were plated at the density of 1 × 10⁶ cells/mL. The cells were transfected with 1.5 µg/mL DNA or RNA by using calcium phosphate transfection method. Twenty-four hours later, the cells were split to 1 × 10⁶ cells/mL per treatment with 20-hydroxyecdysone at 1 µM for 24 h. Cells were then stimulated with peptidoglycan (Invivogen) for 5 h for Northern blots, up to 10 min for Relish cleavage and phosphorylation, 15 min for nuclear translocation and ChIP experiments. For stable cell lines, pPacPL constructs with actin promoter were transfected into S2* cells in conjunction with pH5-neo, 50:1; stable transfecants were then selected with G418 (Gibco; 800 µg/mL).

RNA and Protein Analysis. For Northern blot analysis, total RNA was extracted with TRIzol (Invitrogen), transferred to GeneScreen Plus Hybridization Transfer Membrane (Perkin-Elmer) after electrophoresis and blots were hybridized by using ExpressHyb (Clontech) system with radioactive probes. For Western blot analysis, cells or flies were lysed in lysis buffer (20 mM Tris at pH 7.6, 150 mM NaCl, 25 mM β-glycerophosphate, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, 1 mM NaVO₄, and protease inhibitors); total protein extracts were separated by SDS/PAGE and transferred to PVDF membrane. Antibodies used for immunoprecipitation or immunoblotting were as follows: anti-FLAG (Sigma) and anti-V5 (Sigma). A phosphospecific antibody against Relish SS528/529 was produced by immunizing rabbits with the Ac-FRKLIEHN(p-S)(pS)DLEKIC-amide (520–535) phosphopeptide. The polyclonal sera was then negatively selected through non-phosphopeptide column 2 times, followed by binding and eluting from phosphopeptide column (Quality Controlled Biochemicals).

Kinase Assays. Wild-type rRelish, wild-type, and kinase-dead (K50A) IKKβ proteins were baculovirus expressed by using Bac-to-Bac Baculovirus Expression Systems (Gibco) as previously described (2, 3). SF9 cells were infected with viral stocks that express His-tagged proteins for 3 days at 27 °C. Cell pellets were lysed in lysis buffer [50 mM Tris, pH 8.5, 1% Nonidet P-40, 5 mM 2-mercaptoethanol, 1× protease inhibitor mixture (Sigma), 1 mM PMSF] and sonicated. The lysates are centrifuged and the supernatant is incubated with Ni-beads (Qiagen) for 2 h rocking at 4 °C. The beads were washed 5 times with wash buffer [20 mM Tris, pH 7.9, 0.5 M NaCl, 20 mM imidazole, 5 mM 2-mercaptoethanol, and 1× protease inhibitor mixture (Sigma)]. The slurry was loaded on an empty column and proteins were eluted by using elution buffer [20 mM Tris, pH 7.9, 0.5 M NaCl, 0.25 M imidazole, 5 mM 2-mercaptoethanol, and 1× protease inhibitor mixture (Sigma), 1 mM PMSF]. The eluates were dialyzed against the dialysis buffer (10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM DTT, 10% glycerol, 1 mM PMSF). Wild-type Relish and IKKβ proteins were then loaded on a Mono Q column for a second step of purification. Fractions were collected with 10–60% salt gradient of buffer B (20 mM Tris, pH 8.0, 1 mM DTT, 10% glycerol, 1 mM PMSF, and 1 M NaCl). The fractions were analyzed by Coomassie staining, Western blot, and kinase assays. Appropriate fractions were pooled, and their concentrations were determined by Coomassie staining with BSA standards. Also, WT and SS528/529AA mutant Relish were translated in vitro in reticulocyte lysates (Promega) and immunoprecipitated by using anti-FLAG agarose (Sigma). One-third of these immunoprecipitates was used in the control Western blot, whereas the rest was used in an in vitro kinase reaction with recombinant Drosophila IKKβ and [γ-32P]ATP (3). Kinase reactions were performed in kinase buffer (20 mM Hepes at pH 7.6, 20 mM β-glycerophosphate, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT, 0.1 mM NaVO₄, 200 µM ATP, and 5 µCi of [γ-32P]ATP).

Phosphoamino Acid Analysis. In vitro-phosphorylated Relish was separated by SDS/PAGE, transferred to PVDF membrane, excised, and incubated with 6 M HCl at 110 °C for 1 h. The resulting amino acids were applied to TLC plates with cold phosphoamino acid standards. Phosphoamino acids were separated from each other by electrophoresis in 2 dimensions: 20 min at 1.5 kV in pH 1.9 buffer (formic acid, 25%; acetic acid, 78%) followed by 16 min at 1.3 kV in pH 3.5 buffer (pyridine, 0.5%; acetic acid, 5%). Phosphoamino acids were visualized by autoradiography, and radioactive spots were aligned with cold phosphoamino acid marker spots.

Confocal Microscopy. Cell lines stably expressing YFP–Relish-WT and -SS528/529AA were selected with G418 selection. For confocal microscopy, the stable cells were treated for 24 h with 20-hydroxyecdysone, and then were plated on Con A-treated 35-mm glass-bottomed culture dishes and were visualized by fluorescence microscopy with the 63× objective of a Leica SP2 AOB laser-scanning microscope before and 10 min after peptidoglycan stimulation. Nuclei were stained with Hoechst 34580 (Invitrogen) and images were produced by sequential scanning with 514-nm laser excitation and a 522- to 599-nm emission window for YFP and 405-nm laser excitation and a 523- to 600-nm emission window for Hoechst 34580.

DNA Affinity Purification Assay. Biotinylated wild-type Dipterica kb oligo (biotin-5′-CATCGGGGATTCCTTTT-3′), nonbiotinylated wild-type oligo (5′-CATCGGGGATTCCTTTT-3′), and biotinylated mutant oligo (biotin-5′-CATCGGGGATTCCTTTT-3′) were custom made. Oligonucleotides were annealed with the corresponding antisense oligonucleotide in 1× STE buffer, containing 10 mM Tris-HCl, pH 8, 50 mM NaCl, and 2 mM EDTA. Double-stranded oligonucleotides were mixed with 600 µg of nuclear extracts in 500 µL of binding buffer containing 20 mM Tris-HCl, pH 7.5, 75 mM KCl, 1 mM DTT, and 5 mg/mL BSA in presence of 13% glycerol and 20 µg of poly(dI-dC) and incubated 1 h at...
4 °C. Then streptavidin magnetic beads (Promega) were added to the reaction mixture and incubated for 1 h at 4 °C. The beads were collected with a magnet and washed 3 times with 500 μL of binding buffer. The bound proteins were eluted from the beads by boiling in sample buffer and were resolved on 8% SDS/PAGE followed by immunoblotting with anti-FLAG antibody (Sigma).

**Chromatin Immunoprecipitation.** Stable cell lines expressing Relish wild-type or SS528/529AA were treated with 1 μM 20-hydroxyecdysone and stimulated with peptidoglycan for 15 min. Cells were cross-linked with 1% formaldehyde for 10 min at room temperature and quenched with 120 mM glycine. A total of 5 × 10^6 cells was used for each immunoprecipitation. Cells were harvested and spun at 700 × g for 5 min, washed 2 times with cold PBS, resuspended in 600 μL sonication buffer [10 mM Tris, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1× protease inhibitor mixture (Sigma)] and sonicated 5 times 10 s with 1-min intervals at 30% power by using Fisher Scientific Model 500 Sonic Dismembrator. Sonicated lysates were centrifuged at 8,500 × g for 10 min and protein–DNA complexes were immunoprecipitated in IP buffer (0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris 8.0, 1× protease inhibitor complex, 1 mg/mL BSA) overnight at 4 °C by using 8WG16 (Covance) for RNA polymerase II recruitment or anti-FLAG agarose (Sigma) for Relish–DNA binding. Wash beads 3 times 5 min with low salt buffer (0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 150 mM NaCl, 20 mM Tris 8.1, 2 mM EDTA, 1× protease inhibitor mixture), 3 times 5 min with high salt buffer (0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 500 mM NaCl, 20 mM Tris 8.1, 2 mM EDTA, 1× protease inhibitor mixture), 2 times 5 min with lithium buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 10 mM Tris 8.1, 1 mM EDTA, 1× protease inhibitor mixture), and 2 times 5 min with Tris-EDTA (TE) buffer. Protein–DNA complexes were eluted in 2 times 125 μL elution buffer (50 mM NaHCO3, 1% SDS). A total of 15 μL of 5M NaCl was added to 250 μL of eluates and de-cross-linked overnight at 65 °C. Eluates were column purified and analyzed by qPCR, with a series of primers across the Dipteracin locus (−1.8 to +5.5 kb). For analysis for each PCR product, the signal was first normalized relative to the input DNA levels, and then the fold enrichment was calculated by dividing the normalized signal from the stimulated sample with the normalized signal from the unstimulated sample (4).

**Fig. S1.** C-terminal phosphoacceptor sites of Relish are not required for phosphorylation and cleavage. (A) Wild-type and mutant versions of Relish were translated in vitro, immunoprecipitated with FLAG antibody, and used as a substrate in kinase reaction with recombinant *Drosophila* IKKβ and [γ-32P]ATP. One-third of immunoprecipitated translation reaction was immunoblotted with FLAG antibody to confirm translation and immunoprecipitation (Lower). Wild-type and mutant Relish [with the 10 phosphoacceptor residues in C-terminal 107-aa region changed to alanine: S871A, S872A, T875A, S897A, S907A, T921A, S946A, S950A, T951A, T971A (6SA4TA)] were both robustly phosphorylated. (B) Phosphoacceptor sites in the C terminus of Relish are not required for peptidoglycan-induced cleavage. Cleavage of wild-type and mutant Relish proteins, in stably transfected *Drosophila* S2*+* cells, was analyzed by immunoblotting by using FLAG antibody before and after peptidoglycan stimulation.
IKK-mediated phosphorylation of Relish occurs primarily on serine residues. Relish was in vitro phosphorylated by IKKβ and hydrolyzed with 6N HCl. The resulting amino acids were separated by electrophoresis in 2 dimensions. The cartoon on the left demonstrates the expected positions of phosphoserine (pSer), phosphothreonine (pThr), phosphotyrosine (pTyr), inorganic phosphate (Pi), and peptide products of partial digestion after acid hydrolysis [Hardie DG (1999) Protein Phosphorylation: A Practical Approach (Oxford Univ Press, Oxford)].
Fig. S3. 2D phosphopeptide mapping identifies a signal-induced phosphopeptide dependent on IMD pathway components. Relish phosphopeptides were analyzed by 2D phosphopeptide mapping by using S2* stable cell lines expressing wild-type FLAG-tagged Relish, as in Fig. 1. The single phosphopeptide spot that appeared after PGN stimulation (A and B) was still present after LacZ RNAi treatment, but it was not detected in cells treated with RNAi targeting TAK1 or TAB2 (A), or DIAP2 or DREDD (B). The PGN-induced phosphopeptide spot was also detected in stable cell lines that express noncleavable D545A Relish (C).
Fig. S4. Catalytically active DREDD specifically cleaves Relish. (A) V5-tagged DREDDWT, catalytically inactive DREDDC-A, and Dronc, another Drosophila apical caspase, were overexpressed by using a copper inducible promoter. Lysates were analyzed by immunoblotting for Relish cleavage. Overexpressed DREDDWT but not the catalytically inactive DREDDC-A or Dronc led to Relish cleavage. All transgenes were expressed (Lower). (B) Active drICE, a Drosophila effector caspase, cleaved PARP but not Relish.
Fig. 55. Relish serines S28/S29 are not required for nuclear translocation or DNA binding. (A) Nuclear translocation of wild-type and S5528/S529AA mutant Relish proteins after peptidoglycan stimulation was analyzed by confocal microscopy. Mutant Relish was translocated similar to wild type, suggesting that phosphorylation of these serine residues is not required for nuclear translocation of Relish. (B) DNA binding was analyzed by ChIP assay on chromatin from S2* cells stably expressing FLAG-tagged wild-type or S5528/S529AA mutant versions of Relish, using FLAG monoclonal antibody. Both wild-type and S5528/S529AA mutant version of Relish bound equally. Also, S5528/S529AA mutant bound to the xB element from the Diptericoin promoter in an in vitro biotin-oligo pull-down assay. NB-Oligo, nonbiotinylated wild-type oligo used as competitor. BM-Oligo, biotinylated mutant oligo used as a negative control.