The antibacterial arm of the *Drosophila* innate immune response requires an IκB kinase

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The *ird5* gene was identified in a genetic screen for *Drosophila* immune response mutants. Mutations in *ird5* prevent induction of six antibacterial peptide genes in response to infection but do not affect the induction of an antifungal peptide gene. Consistent with this finding, *Escherichia coli* survive 100 times better in *ird5* adults than in wild-type animals. The *ird5* gene encodes a *Drosophila* homolog of mammalian IκB kinases (IKKs). The *ird5* phenotype and sequence suggest that the gene is specifically required for the activation of Relish, a *Drosophila* NF-κB family member.

[*Key Words*: Innate immunity; IκB kinase; *Drosophila*; antimicrobial peptide; Relish; NF-κB]

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In both mammals and *Drosophila*, microbial infection activates Toll-like receptor (TLR) signaling pathways as a part of the innate host defense response (for review, see Anderson 2000). TLR-mediated signaling pathways are essential for appropriate responses to bacterial infection. In addition, mouse Tlr4 mediates septic shock associated with infection by gram-negative bacteria (Vogel 1992; Poltorak et al. 1998).

The available data indicate that different microbial cell wall components activate different Toll-like receptor signaling pathways, which regulate distinct sets of target genes. In mammals, TLR4 is the prime mediator of responses to bacterial lipopolysaccharide, while TLR2 mediates responses to bacterial peptidoglycans (Poltorak et al. 1998; Takeuchi et al. 1999; for review, see Beutler 2000). The best-studied aspect of the *Drosophila* innate immune response is the rapid transcriptional induction of antimicrobial peptide genes in response to infection (Hultmark 1993; Hoffmann 1995). Infection by different classes of microorganisms leads to the preferential induction of particular subsets of antimicrobial peptides (Lemaitre et al. 1997), indicating that different microbial components activate different signaling pathways.

At least two Toll-related signaling pathways are required for the activation of the *Drosophila* antimicrobial peptide genes. The Toll pathway itself, which was first identified because of its essential role in *Drosophila* embryonic patterning (Anderson et al. 1985), is essential for the induction of an antifungal peptide gene, *drosomycin*, although the antibacterial peptide genes are still induced in Toll pathway mutants (Lemaitre et al. 1996). Another *Drosophila* member of the Toll family, 18-wheeler, is required for the normal induction of *attacin*, an antibacterial peptide gene, but mutations in *18-wheeler* do not prevent the induction of other antibacterial peptides (Williams et al. 1997). The *imd* gene is important for the induction of Diptericin and other antibacterial peptides (Lemaitre et al. 1995a; Corbo and Levine 1996) and, therefore, appears to be a component of a third signaling pathway activated by infection, but its biochemical function is not known.

Each of the three *Drosophila* signaling pathways activated by infection leads to activation of NF-κB/Rel dimers, just as the mammalian TLRs activate NF-κB. All three *Drosophila* Rel proteins, Dorsal, Dif, and Relish, are expressed in the fat body cells that produce the antimicrobial peptides, and all three are activated within 30 min after infection by translocation from the cytoplasm to the nuclei (Ip at al. 1993; Lemaitre et al. 1995b; Stöven et al. 2000). Adults that lack Dif fail to induce Drosomycin, an antifungal peptide, and Defensin, which is active against gram-positive bacteria, but the other antimicrobial peptide genes are induced normally (Manfruelli et al. 1999; Meng et al. 1999; Rutschmann et al. 2000). Animals that lack Dorsal show normal induction of the antimicrobial peptide genes in response to infection (Lemaitre et al. 1995b), although Dorsal may act redundantly with Dif in larvae (Manfruelli et al. 1999; Rutschmann et al. 2000). Relish is a compound protein with an N-terminal Rel domain and a C-terminal IκB-like domain, similar to mammalian p100 and p105 (Dushay et al. 1996). Relish is activated by signal-depen-

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dent proteolysis, which liberates the N-terminal Rel domain, allowing it to translocate into nuclei (Stöven et al. 2000). Adults that lack Relish completely fail to induce the antibacterial peptides Diptericin and Cecropin and show reduced induction of the other antimicrobial peptides (Hedengren et al. 1999).

The signaling pathway that activates Relish and controls induction of the antibacterial peptide genes has not been defined. We carried out a genetic screen to identify EMS-induced mutations on the *Drosophila* third chromosome that affect the antibacterial signaling pathway (Wu and Anderson 1998). A large number of mutants were identified and named *ird* (immune response deficient) mutants. This screen identified two alleles of the *ird5* gene on the basis of the failure of homozygous mutant larvae to induce a *diptericin-lacZ* reporter gene in response to infection. Here we show that the *ird5* gene is essential for antibacterial responses and encodes a *Drosophila* homolog of mammalian IκB kinases.

Results and Discussion

ird5 *mutations block the antibacterial but not the antifungal immune response*

Mutations in *ird5* prevent induction of a *diptericin-lacZ* reporter gene in response to infection and also prevent transcriptional induction of the endogenous *diptericin* gene (Wu and Anderson 1998). We examined *E. coli*-induced expression of all seven classes of antimicrobial peptide genes in *ird5* mutant larvae by RNA blot hybridization, including the genes encoding antibacterial (Diptericin, Cecropin A, Defensin, Attacin, Drosocin, and Metchnikowin) and antifungal (Drosomycin) peptides (Fig. 1A,B). In wild-type larvae, all the antimicrobial genes were strongly induced after bacterial challenge. In contrast, in larvae homozygous for either *ird5* allele, there was no detectable induction of the *diptericin*, *cecropin A*, *defensin*, *drosocin*, or *metchnikowin* genes. The *attacin* gene was induced in the mutants to ~30% of normal levels, while *drosomycin* was induced to normal levels (Fig. 1; Table 1). The same effects on the induction of antimicrobial peptide genes were seen in $ird5^1/Df$ and $ird5^2/Df$ animals (data not shown), suggesting that both alleles cause a complete loss of gene function.

Mutations in three other genes, *imd*, *Relish*, and *Dredd*, have been shown to prevent normal induction of antibacterial peptide genes in adult *Drosophila* (Hedengren et al. 1999; Elrod-Erickson et al. 2000; Leulier et al. 2000). We compared the pattern of antimicrobial peptide gene induction in *ird5* mutants with that in *imd* and *Relish* mutants in both larvae and adults (Fig. 1A; Table 1). Mutations in all three genes had very similar effects on antimicrobial gene induction in larvae: *diptericin* and *cecropin A* were not induced; *attacin* induction was reduced and *drosomycin* induction was normal. In adult animals, the antimicrobial gene expression phenotype of *ird5* and *Relish* mutants were very similar: *diptericin* induction was blocked, *cecropin A* and *attacin* induction was reduced, and *drosomycin* induction was normal. The antimicrobial gene expression phenotype of *imd* adults was slightly different, with some residual *diptericin* expression. Mutations in *Dredd*, a *Drosophila* caspase, prevent normal induction of *diptericin* and *attacin* and allow induction of *drosomycin* (Elrod-Erickson et al. 2000; Leulier et al. 2000). These comparisons suggest that *ird5*, *Dredd*, *Relish*, and probably *imd* act in the same signaling pathway to control the induction of antibacterial peptide genes in response to infection.

To assess the importance of the *ird5* gene in controlling the growth of invading bacteria, we compared bacterial survival and growth in wild-type, *ird5*, *imd*, and *Relish* animals (see Materials and Methods). In wild-type larvae, most of the *E. coli* injected into the animal were killed by 6 h after infection (Fig. 2A). At this same time point, there were four to 15 times as many *E. coli* in *ird5* mutant larvae as in wild-type animals (Fig. 2B). The effects of the *ird5* mutations were more striking in experiments with adults: at 24 h after infection, there were 20–350 times as many bacteria per animal in *ird5* mutants compared to wild type. The bacterial growth phenotype of *ird5* mutants was similar to that seen in *Relish*

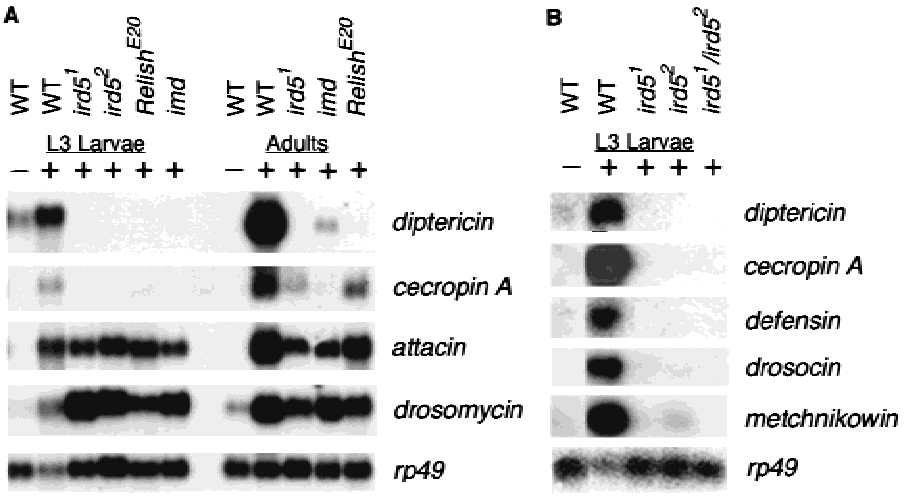

Figure 1. Expression of the antimicrobial peptide genes in wild-type and mutant animals. The induction of antimicrobial peptide genes was assayed by Northern blot. (*A*) Induction of *diptericin*, *cecropin*, *drosomycin*, and *attacin* in wild-type, *ird5*, *Relish*, and *imd* mutants in response to infection at L3 larval and adult stages. (*B*) Induction of the battery of antimicrobial peptides in wild-type and *ird5* mutants at L3 larval stage. Each lane contains 30 μg of total RNA from untreated third-instar larvae (–) or 2–3 h after injection with *Escherichia coli* (+). *rp49* was used as loading control.

Table 1. *Induction of antimicrobial peptides in mutants*

Antimicrobial peptides	L3 Larvae						Adults				
	Wild type	Wild type	*ird5*^{1}	*ird5*^{2}	*Relish*	imd	Wild type	Wild type	*ird5*^{1}	*Relish*	*imd*
	Uninfected	Infected					Uninfected	Infected			
diptericin	0	100	0 ± 0	0 ± 0	0 ± 0	0 ± 2	0	100	1 ± 1	0 ± 0	13 ± 7
cecropin	0	100	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0	100	14 ± 2	30 ± 7	5 ± 3
attacin	0	100	33 ± 20	29 ± 16	43 ± 28	20 ± 15	0	100	35 ± 17	64 ± 30	47 ± 26
drosomycin	0	100	125 ± 21	54 ± 39	46 ± 28	76 ± 30	0	100	40 ± 15	71 ± 18	90 ± 8

Induction of antimicrobial peptides in response to infection in mutant animals, expressed as the percentage of the induction seen in wild type. Northern blots (including the data in Fig. 1) were analyzed by phosphorimager and normalized relative to the *rp49* loading control. The average and standard deviation in three experiments are shown.

mutant larvae and adults and somewhat stronger than that of *imd* mutants (Fig. 2B). This is consistent with the stronger effects of *ird5* and *Relish* on the antimicrobial peptide genes: Mutations in either *ird5* or *Relish* prevent normal induction of *diptericin*, *cecropin*, *drosocin*, *attacin*, and *metchnikowin* (Fig. 1; Hedengren et al. 1999), while induction of *metchnikowin* is induced in *imd* mutants (Levashina et al. 1998).

ird5 *encodes a* Drosophila *IκB kinase*

We mapped the mutations responsible for the failure to induce the *diptericin-lacZ* reporter gene for both *ird5* alleles between the visible markers *cu* (86D1-4) and *sr* (90D2-F7) on the right arm of the third chromosome. The deficiency Df(3R)*sbd*^{45}(89B4-10) failed to complement the immune response defect of either *ird5* allele. Further deficiency-complementation tests and male recombination mapping narrowed the *ird5* interval to 89B4-9, between *pannier* and *Stubble* (data not shown). Two molecular-defined genes in this interval were considered as candidates responsible for the *ird5* phenotype, *Akt* and a gene defined by an EST that was related to mammalian IκB kinases (IKKs; Fig. 3A). Mutant alleles of *Akt* cause recessive lethality (Staveley et al. 1998), and *ird5*/*Akt*(*l(3)89Bq)* heterozygous animals were viable and showed normal induction of the *diptericin-lacZ* reporter gene (data not shown), indicating that the *ird5* phenotypes were not caused by mutations in *Akt*.

Mammalian IKKβ is required for activation of NF-κB

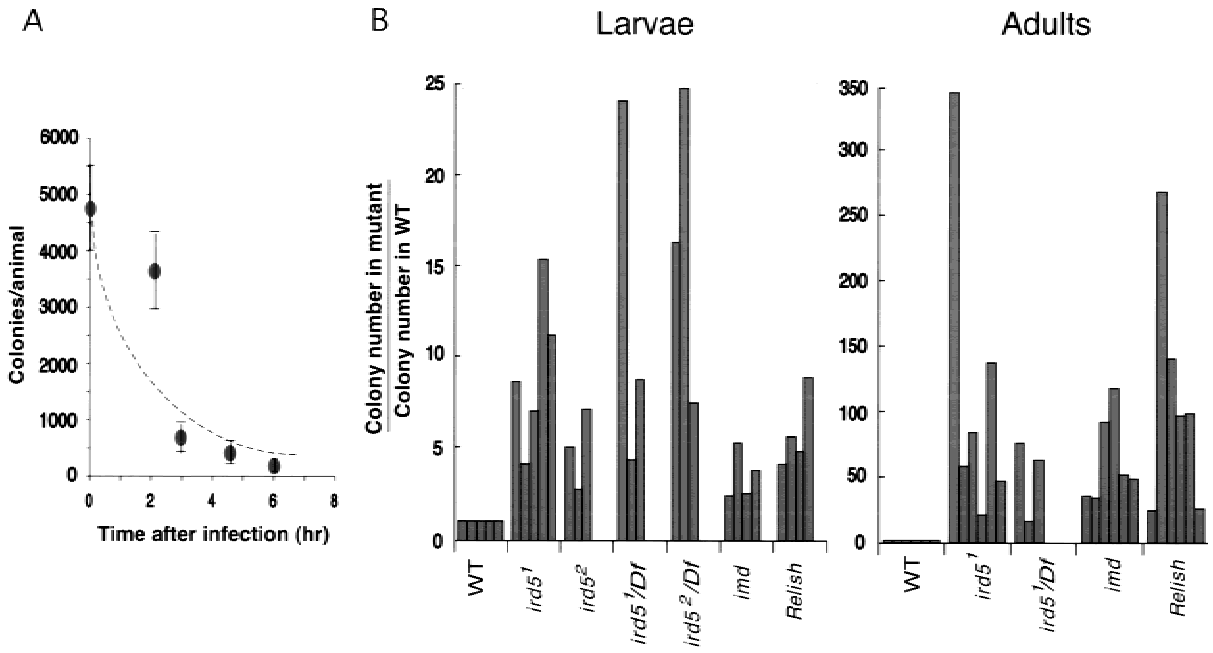

Figure 2. Analysis of bacterial growth after *Escherichia coli* infection. (*A*) Wild-type larvae kill *E. coli* within hours after infection. The number of ampicillin-resistant *E. coli* present per animal at time points after injection is shown. The data shown are the average of two experiments, with three to five animals per time point in each experiment. (*B*) Bacterial growth in wild-type, *ird5*, *Relish*, and *imd* mutant larvae and adults after infection, indicated as the ratio of the number of colony-forming units (cfu) per animal in the mutant relative to the number of cfu in the wild-type control in the same experiment. Each bar represents an independent experiment. For larvae, the number of bacteria per animal was assayed at 6 h after infection to ensure that the animals had not begun to pupate. For adults, the number of bacteria per animal was assayed at 24 h after infection. Df: Df(3R)*sbd*^{45}.

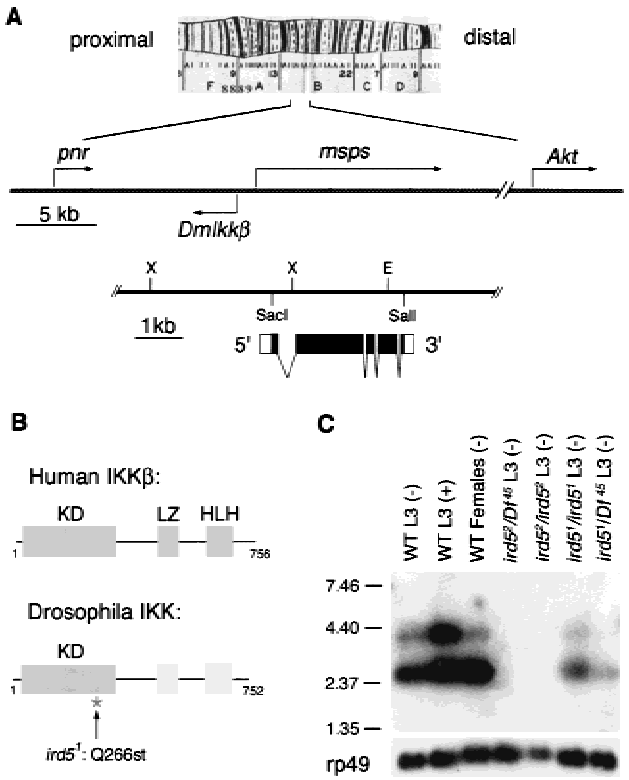

Figure 3. The wild-type *DmIkk*β gene and the *ird5* mutations. (*A*) The *DmIkk*β genomic region in the polytene map. Based on deficiency mapping, *ird5* lies in 84B4-9, proximal to *Sb*; male recombination mapping placed *ird5* distal to *pannier* (*pnr*; data not shown). In genomic DNA, *DmIkk*β lies between *pnr* and *mini spindles* (*msps*; Cullen et al. 1999); *Akt* is 50 kb distal to *DmIkk*β. Comparison of the genomic and cDNA sequences indicated that *DmIkk*β has five exons that produce a 2.7-kb transcript, shown relative to the restriction map of genomic DNA. (*B*) Comparison of domains of *ird5*/*DmIkk*β and human *IKK*β indicating the position of the *ird5*^{1} stop codon. The homology between the *Drosophila* and human genes is greatest in the kinase domain (34% identity to human *IKK*β); there is weak homology to the leucine zipper (LZ) and helix–loop–helix (HLH) motifs in the *Drosophila* gene (dotted boxes). (*C*) *DmIkk*β expression in wild-type and *ird5* mutants, as assayed by Northern blot. Two transcript sizes, 2.7 and 4.2 kb, are present in wild-type animals; the smaller transcript corresponds to the cDNAs we have isolated. Based on phosphorimager analysis, both *DmIkk*β transcripts are induced 1.5-fold at 2 h after infection compared with uninfected animals. *rp49* was the loading control. (–), uninfected; (+), 2 h after *Escherichia coli* infection; L3, third-instar larvae; F, adult female; Df, Df(3R)*sbd*^{45}.

in response to inflammatory signals such as TNF-α and IL-1 (Q. Li et al. 1999; Z. Li et al. 1999); the IKK homolog was therefore considered as a candidate gene for *ird5*. We cloned a full-length cDNA for the IKK homolog, which we call *DmIkk*β (Fig. 3B). The same gene was also identified molecularly as encoding a kinase activated by LPS in a *Drosophila* cell line (Kim et al. 2000; Medzhitov and Janeway 2000; Silverman et al. 2000). Based on genomic DNA sequence, *DmIkk*β is located between *pannier* and *mini-spindles* (Fig.3A; Experimental Procedures). We detected two size classes of transcripts, 2.7 and 4.2 kb, from the *DmIkk*β gene (Fig. 3C); the cDNA corresponded to the 2.7-kb transcript. Both transcripts were expressed at higher levels after infection (Fig. 3C). Similar induction of other genes that encode components of the immune response machinery has been observed previously (Dushay et al. 1996; Lemaitre et al. 1996). We sequenced the complete open reading frame of *DmIkk*β from the *ird5*^{1} and *ird5*^{2} chromosomes. We identified a single C-to-T nucleotide substitution in *ird5*^{1} that would change a glutamine codon (CAA) at amino acid 266 of the open reading frame to a stop codon (TAA) within the conserved kinase domain (Fig. 3). No sequence changes were identified in the open reading frame in *ird5*^{2}; however, neither *DmIkk*β transcript was detectable in *ird5*^{2} homozygotes (Fig. 3). This analysis indicates that both *ird5* alleles are associated with mutations that should abolish *DmIkk*β activity.

To confirm that *ird5* is the same as the *DmIkk*β gene, we tested the ability of a *DmIkk*β transgene to rescue the immune response defect of *ird5* flies. Neither a UAS-*DmIkk*β cDNA transgene nor a fat-body GAL4 driver alone rescued the immunity phenotype of *ird5*^{1} (Fig. 4). However, in *ird5*^{1} *diptericin-lacZ*/Df(3R)*sbd*^{45} larvae carrying both a UAS-*DmIkk*β cDNA transgene and a transgene that expressed GAL4 in the fat body, the *diptericin-lacZ* reporter was activated after *E. coli* infection (Fig. 4). These results demonstrate that *DmIkk*β is the gene responsible for the *ird5* mutant phenotype.

Ird5/DmIkkβ *is not a component of the Toll/cactus pathway*

The *ird5* immune response phenotype showed striking specificity: All of the antibacterial peptide genes were strongly affected by the *ird5* mutations, but the antifungal peptide gene *drosomycin* was induced normally in *ird5* mutants. The specific immune response phenotype of *ird5*/*DmIkk*β in vivo contrasts with the global effects on antimicrobial peptide genes seen in cell lines when a dominant negative form of the same gene was expressed in cultured cells (Kim et al. 2000). The *ird5*/*DmIkk*β mutant phenotype implies that, in vivo, *ird5* is not an essential component of the Toll pathway, which is required for the induction of *drosomycin*. The *ird5*/*DmIkk*β gene is therefore a component of an independent signaling pathway, which could be activated by another member of the *Drosophila* Toll-like receptor family (Tauszig et al. 2000).

Mammalian IKKα and IKKβ phosphorylate serine residues in the N-terminal domain of IκB that target IκB for degradation, thereby allowing the nuclear localization and activation of NF-κB (Chen et al. 1996; DiDonato et al. 1997; Regnier et al. 1997). The *ird5*/*DmIkk*β sequence suggests that the protein encoded by this gene phosphorylates an IκB-like protein. There are two known *Drosophila* IκB-like proteins that could act as inhibitor proteins in the immune response, Cactus, and the C-terminal ankyrin repeat domain of Relish. In *cactus* mutants, *drosomycin* is expressed constitutively, but the

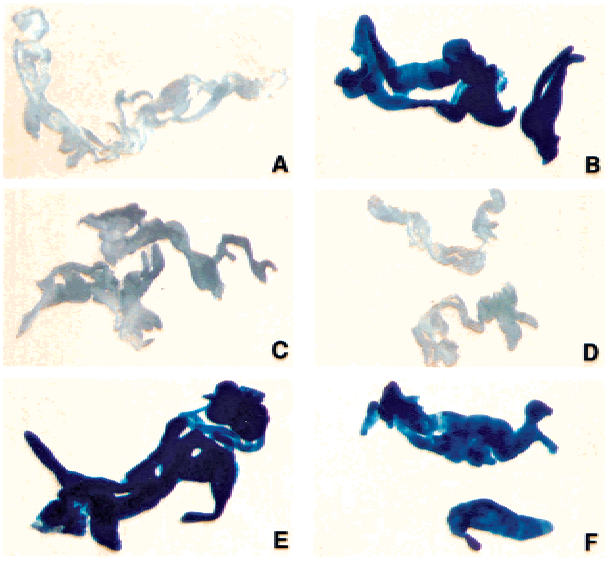

Figure 4. Expression of the *DmIkk*β cDNA rescues the *ird5* phenotype. Each panel shows histochemical staining of fat body β-galactosidase activity in third-instar larvae carrying the *diptericin-lacZ* reporter gene. Wild-type uninfected (*A*) and infected (*B*) larvae, showing the normal induction of the reporter after infection. (*C*) Fat body from infected *w*; Gal-c564/+; *ird5*^{1}, *diptericin-lacZ*, *e*/Df(3R)*sbd*^{45}, *e* larvae. These *ird5* animals carrying the GAL4 driver failed to induce the reporter gene, just as do *ird5* animals. (*D*) Fat body from infected *w*; UAS-*DmIkk*β-cDNA (6–4)/+; *ird5*^{1} *diptericin-lacZ*, *e*/ *ird5*^{1} *diptericin-lacZ*, *e* larvae. These *ird5* animals carrying the UAS-cDNA failed to induce the reporter gene, like *ird5* mutants. (*E*) and (*F*) *ird5* homozygous larvae carrying both the GAL4 driver and the UAS-DmIkkβ transgene expressed the reporter gene after infection. (*E*) Fat body from infected *w*; UAS-*DmIkk*β-cDNA (6–4)/Gal-c564 ; *ird5*^{1} *diptericin-lacZ*, *e*/Df(3R)*sbd*^{45}, *e* larvae. (*F*) Infected larvae carrying a different transgene insertion site also express the reporter: fat body from infected *w*; UAS-*DmIkk*β-cDNA (9–9)/Gal-c564; *ird5*^{1} *diptericin-lacZ*, *e*/Df(3R)*sbd*^{45} animals.

antibacterial peptide genes are not (Lemaitre et al. 1996), which indicates that Cactus is not involved in the pathways that regulate the antibacterial peptide genes. Furthermore, *ird5*/*DmIkk*β homozygous mutant females are fertile, demonstrating that this gene is not required for degradation of Cactus during dorsal-ventral patterning in the embryo.

Ird5/DmIkkβ *and* Relish *act in a common pathway*

The *ird5*/*DmIkk*β phenotype is similar to the phenotype of *Relish* mutants (Hedengren et al. 1999; Figs. 1,2). For both genes, homozygous mutant flies are viable and fertile, indicating that the two genes are not essential for development. Mutations in either *Relish* or *ird5*/*DmIkk*β completely prevent induction of *diptericin* and *cecropin* but allow some induction of *attacin* and *drosomycin* (Fig. 1). Mutations in either gene produce comparable effects on bacterial growth (Fig. 2). These results argue that *ird5*/*DmIkk*β and *Relish* act in the same signaling pathway and suggest that Ird5/DmIkkβ activates Relish-containing dimers. Relish activation requires proteolytic cleavage of Relish protein into an N-terminal Rel domain that translocates to the nucleus and a C-terminal ankyrin repeat domain that remains in the cytoplasm (Stöven et al. 2000). Recent biochemical experiments have shown that DmIkkβ can phosphorylate Relish protein (Silverman et al. 2000), which is consistent with the model that phosphorylation of Relish by DmIkkβ leads to targeted proteolysis and activation of Relish.

Drosophila *IκB kinases in development and immunity*

Although *ird5*/*DmIkk*β is expressed maternally (data not shown), *ird5* mutant females are fertile, demonstrating that the gene is not required for embryonic dorsal-ventral patterning. However, a small fraction of embryos (~0.5%) produced by homozygous *ird5*^{1} or *ird5*^{1}/*ird5*^{2} females show a weakly dorsalized phenotype (Fig. 5), suggesting that *ird5*/*DmIkk*β does have a minor role in the maternal pathway that activates Dorsal. We suggest that there is another kinase in the early embryo that is primarily responsible for phosphorylation and degradation of Cactus. The normal induction of *drosomycin* in *ird5*/*DmIkk*β mutants suggests that there will also be another kinase activated by the Toll pathway in the immune response—perhaps the same kinase that acts downstream of Toll to activate Dorsal in the embryo. The genome sequence indicates that there is one additional IκB kinase gene in *Drosophila*. Future experiments will test whether this gene plays a role in embryonic patterning and the antifungal immune response.

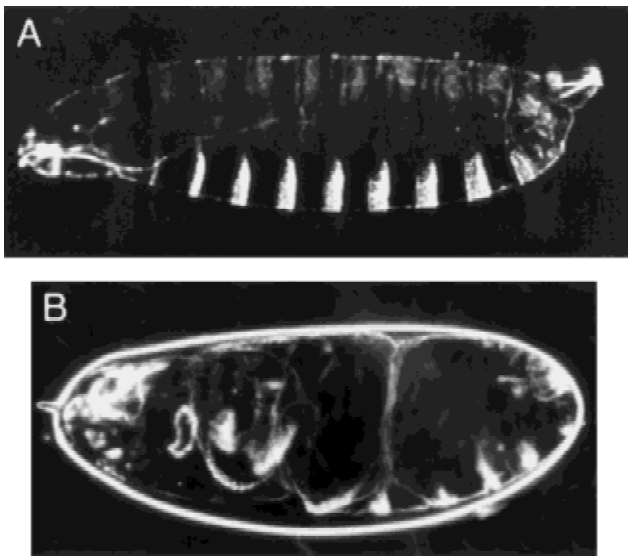

Figure 5. Weak maternal effect of *ird5*. (*A*) Dark field view of the cuticle of a wild-type larva just after hatching. Most of the embryos produced by *ird5* homozygous females show this wild-type cuticle pattern. Dorsal is up. (*B*) Cuticle of a weakly dorsalized embryo produced by an *ird5*^{1}/*ird5*^{1} female, showing the twisted body characteristic of embryos that have deficiencies in mesoderm, the most ventral cell type (Anderson et al. 1985).

Our data suggest that different *Drosophila* Rel dimers are activated by homologous but distinct signaling pathways. Given the similarities of innate immune response pathways in *Drosophila* and mammals, it is likely that similar pathway-specific signaling components will mediate the activities of the members of the mammalian Rel proteins.

Materials and methods

Bacterial infection and bacterial survival in infected animals

In bacterial growth experiments, *E. coli* were introduced into animals by injection, as described previously (Wu and Anderson 1998). For larvae, *E. coli* containing an Amp^r plasmid were diluted 10-fold from an OD_{600} 0.5 culture in PBS and mixed with 1:200-fold diluted India ink. Wandering third instar larvae of the appropriate genotype were injected, with the ink as indicator of the volume injected, transferred to apple juice agar plates, and incubated at 25°C for 6–7 h. Because many of the injected larvae pupated shortly thereafter, longer time points were not assayed. For adults, the *E. coli* culture was diluted 100-fold from an OD_{600} 0.5 culture in PBS; a lower concentration of *E. coli* was used in the adult experiments because adults do not control bacterial growth as efficiently as larvae. Adult flies (1–2 d old) of the appropriate genotype were injected and incubated at 29°C for 24 h. For both larvae and adults, three to five animals at the appropriate time after *E. coli* injections were homogenized in LB media and spread on LB plates containing Ampicillin (50μg/mL).

Molecular characterization of DmIkkβ

A full-length cDNA corresponding to the 2.7-kb RNA was isolated from an embryonic cDNA library, using the ESTs GM10440 and LD09214 (Genome Systems) as probes. According to the NetStart 1.0 program, the open reading frame is likely to be translated starting at a methionine 20 amino acids upstream of that reported previously (Kim et al. 2000; Medzhitov and Janeway 2000); the position of the stop codon in *ird5*^{1} at position 266 is numbered using this upstream methionine.

We did not isolate cDNAs corresponding to the larger (4.2-kb) RNA, but RT–PCR analysis of RNA from infected larvae revealed that transcripts exist with additional 5′ and 3′ sequences. One transcript has an alternative 3′ coding exon of five amino acids and a different 3′ untranslated region than present in the cDNA.

For sequencing, genomic DNA from wild-type and homozygous *ird5* mutant flies was amplified by PCR. Two independent clones of each PCR product were subcloned into pGEM-T vector (Promega) and were sequenced by the Cornell sequencing facility. For Northern analysis of the transcript, RNA was prepared using the RNA-STAT60 (Tel-Test) reagent. Poly(A)$^+$ RNA was purified using the Oligotex (QIAGEN) kit. Four micrograms of poly(A)$^+$ RNA was electrophoresed and transferred to Hybond N+ nylon filters.

Northern analysis

To assay antimicrobial peptide gene induction, *E. coli* were injected into wandering third-instar larvae and adult flies, as described previously (Wu and Anderson 1998). DNA from the complete *DmIkk*β cDNA and antimicrobial peptide cDNAs were labeled by random priming (Boehringer) and used as probes.

Embryonic phenotypes

Cuticle preparations were made as described previously (Wieschaus and Nüsslein-Volhard 1986).

Transgene production and rescue of the ird5 phenotype

The 2.7-kb *DmIkk*β cDNA was cloned into the pUAST (*w*+) transformation vector (Brand and Perrimon 1993). The construct was introduced into *y w* flies by P element–mediated transformation (Spradling 1986). w^+ adults were used to establish several independent lines with insertions located on X, second, and third chromosomes. Several lines with insertions on the second chromosome were crossed with flies carrying *ird5*^{1} mutant allele to obtain flies with the following genotype: *w*; UAS-*DmIkk*β (cDNA); *ird5*^{1} *diptericin-lacZ*, *e*/T(2;3) CyO; TM6B Tb (strain A). The second-chromosome GAL4 line Gal-c564, which is expressed in the fat body (Harrison et al. 1995), was used to drive expression of the UAS construct. We confirmed that Gal-c564 is expressed in the fat body by crossing to a UAS-GFP transgenic line. Flies carrying Gal-c564 were crossed to flies carrying a deficiency uncovering *ird5* to obtain flies with the following genotype: *w*; Gal-c564; Df(3R)*sbd*^{45}, *e*/T(2;3) CyO; TM6B Tb (strain B). Strains A and B flies were then crossed and the progeny Tb^+ larvae were selected and tested for the ability to induce the *diptericin-lacZ* reporter gene in response to infection. *Escherichia coli* infection and β-galactosidase activity analysis were performed as described previously (Wu and Anderson 1998), but the staining reaction was carried out for 30–60 min at 37°C.

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