able conditions, circulate and cause cases of poliomyelitis has important implications for current and future strategies of the World Health Organization (WHO) initiative to eradicate polio worldwide (23). First, the eradication of wild poliovirus, now at an advanced stage (23), must be completed as soon as possible. At the same time, it is imperative that immunity gaps in nonendemic countries are prevented, especially in tropical developing countries where the risk for poliovirus circulation is highest (24). After certification of wild poliovirus eradication, a carefully planned strategy for the orderly cessation of OPV use worldwide should be implemented. Finally, sensitive global poliovirus surveillance must be maintained for the foreseeable future, and emergency stockpiles of poliovirus vaccine established, for use in the event of any recurrent poliovirus transmission from chronic poliovirus excretors (25), a breach in poliovirus containment (25), or circulating VDPV.

References and Notes

2. C. A. de Quadros et al., J. Infect. Dis. 175 (suppl. 1), S37 (1997).
6. Supplementary figures and details of experimental procedures are available on Science Online at www.sciencemag.org/cgi/content/full/1068284/DC1.
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Requirement for a Peptidoglycan Recognition Protein (PGRP) in Relish Activation and Antibacterial Immune Responses in Drosophila

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Components of microbial cell walls are potent activators of innate immune responses in animals. For example, the mammalian TLR4 signaling pathway is activated by bacterial lipopolysaccharide and is required for resistance to infection by Gram-negative bacteria. Other components of microbial surfaces, such as peptidoglycan, are also potent activators of innate immune responses, but less is known about how those components activate host defense. Here we show that a peptidoglycan recognition protein, PGRP-LC, is absolutely required for the induction of antibacterial peptide genes in response to infection in Drosophila and acts by controlling activation of the NF-κB family transcription factor Relish.

In response to infection, Drosophila activates the transcription of a battery of antimicrobial peptide genes in cells of the fat body (the insect analog of the liver). Two major branches of this humoral response have been identified; as in mammals, these responses require NF-κB transcription factors (1). One branch activates antifungal responses and requires the receptor Toll and the NF-κB family transcription factor DiF (2–4). The second branch, which is primarily antibacterial, requires the NF-κB protein Relish, an IκB kinase (IKK), a caspase, a mitogen-activated protein kinase kinase kinase, and the death domain protein Imd (5–11).

We have taken a genetic approach to identifying genes required for the antibacterial response (12, 13). One gene that is absolutely required for the induction of the antibacterial response is ird7 (immune response deficient 7). Two mutations in ird7 identified in an ethyleneethanol sulfonate (EMS) mutagenesis screen (12, 13) prevented the induction of three antibacterial peptide genes, Dpericin, Cecropin, and Defensin, after infection by either Gram-negative or Gram-positive bacteria (Fig. 1, A and B). Three other antimicrobial peptide genes, Ataccin, Metchnikowin, and Drosomycin, also failed to be induced to normal levels. The profile of antimicrobial gene expression observed in the ird7 mutants was similar to that observed in imd, DmIkkβ/ird3, and Relish mutants after bacterial infection, but was distinct from that of Toll and Dif mutants (Fig. 1A). This pattern suggests that ird7 is an essential component of the same signaling pathway that requires imd and Relish, but is not required for the Toll-Dif pathway. Both ird7 mutants are homozygous viable and fertile, and blood cells from ird7 mutants can phagocytose bacteria (14); these findings suggest that ird7 is required specifically for the humoral immune response.

The transcription factor Relish directly activates antibacterial target genes in Drosophila. Relish is a compound protein similar to mammalian p100 and p105 (the precursors of the p52 and p50 subunits of NF-κB), with an NH2-terminal Rel homology and a COOH-terminal ankyrin repeat domain similar to that of the NF-κB inhibitor IκB (15). In response to immune challenge, full-length Relish (REL-110) is endopeptidolytically clipped to generate the NH2-terminal REL-68 fragment, which translocates into the nucleus, and the COOH-terminal REL-49 ankyrin repeat fragment, which remains stable in the cytoplasm (16) (Fig. 1C). In contrast to wild-type animals, no processing of Relish was detected in ird7 mutant larvae (Fig. 1C). The
Blots were hybridized with a radiolabeled probe from the second exon of *Drosophila* chromosome 66F5-67A9 (Fig. 2A). The results indicate that *ird7* Rel domain of Relish failed to translocate to the nucleus, including two genes encoding peptidoglycan recognition protein (PGRP) domains, PGRP-LA and PGRP-LC (18). Peptidoglycan is a strong activator of innate immune responses in insects and mammals, and a PGRP was first identified in a silk moth (*Bombyx*). Later studies have implicated PGRPs in innate immune responses from arthropods to mammals (20, 21).

We identified sequence changes that would disrupt the function of PGRP-LC in both *ird7* alleles. The gene was represented by several expressed sequence tag clones that encode a single splice variant, designated PGRP-LCa. In addition, sequences encoding two additional exons encoding PGRP domains (“x” and “y”) were identified in an intron of PGRP-LC (18). We screened a larval-pupal cDNA library from Berkeley Drosophila Genome Project (LP library) with the probe described above for PGRP-LCa (Fig. 2C).

The induction of other antibacterial peptide genes by these bacteria in *ird7* and *imd* mutants was also similar to that shown in *Dipterocera*, which is common to both splice variants. α-Tubulin was the loading control. β-Tubulin was the loading control.

**Fig. 2.** Molecular identification of the *ird7* gene. (A) Genetic mapping of *ird7*. The *ird7* mutation failed to complement *Df(3L)29A6* but complemented *Df(3L)Rd1-2* and *Df(3L)AC1*. Deficiency breakpoints were defined by single-embryo polymerase chain reaction (PCR) (26). P-elements–induced male recombination mapping (27) placed the *ird7* locus between *boule* and *EP(3)3043*. Bars at bottom indicate the region that could include *ird7*. At all steps of mapping, X-Gal staining was used to monitor induction of *Dpt-lacZ* after *E. coli* infection. (B) Expression of PGRP-LC in wild-type and *ird7* mutants. Polyadenylated RNA (4 μg) prepared from wild-type (*P[w+ Dpt-lacZ]ca*) and *ird7* adults, was loaded in each lane. Blots were hybridized with a radiolabeled probe from the second exon of PGRP-LC, which is common to both splice variants. α-Tubulin was the loading control. (C) Molecular lesions in PGRP-LC in *ird7* mutants. The *ird7* allele is associated with an insertion of 858 bp in a common 5′ exon of PGRP-LC that introduces a stop codon and would generate a truncated cytoplasmic protein of 105 amino acids. The *ird7* allele is associated with a nonsense mutation in the x PGRP domain of the PGRP-LCx isoform, which would truncate this isoform. Light gray bars represent the transmembrane domain. Dark gray bars represent peptidoglycan recognition domains. For cloning of PGRP-LCx, a larval-pupal cDNA library (LP library from Berkeley Drosophila Genome Project) was screened using a random-primed probe for putative exon x (18).
Fig. 3. Both PGRP-LCa and PGRP-LCx isoforms rescue induction of the Dpt-lacZ reporter gene in ird7 mutants. Full-length PGRP-LCa and PGRP-LCx cDNAs were cloned into the pUAST (w’ ) transformation vector and introduced into y w flies by P element–mediated transformation (29). The second chromosome c564-GAL4 line, which is expressed in the fat body and other tissues (30), was used to drive expression of the UAS construct. Flies of indicated genotypes were injected with E. coli, incubated for 6 hours, and assayed for β-galactosidase activity using X-Gal. (A) c564-GAL4/CyO; ird7 Dpt-lacZ/ird7 Dpt-lacZ (no UAS-cDNA) animals did not express the reporter gene. (B) UAS-PGRP-LCx/CyO; ird7 Dpt-lacZ/ird7 Dpt-lacZ (no GAL4 driver) did not express the reporter gene. The same result was obtained for UAS-PGRP-LCa/CyO; ird7 Dpt-lacZ/ird7 Dpt-lacZ animals. (C) c564-GAL4/UAS-PGRP-LCa; ird7 Dpt-lacZ/ird7 Dpt-lacZ expressed the reporter gene at high levels after infection, as did c564-GAL4/UAS-PGRP-LCx; ird7 Dpt-lacZ/ird7 Dpt-lacZ animals (D). The GAL4-driven transgenes also showed a low level of constitutive expression of Dpt-lacZ without E. coli injection: (E) c564-GAL4/UAS-PGRP-LCa; ird7 Dpt-lacZ/ird7 Dpt-lacZ, (F) c564-GAL4/UAS-PGRP-LCx; ird7 Dpt-lacZ/ird7 Dpt-lacZ. In four replications of this experiment, the level of X-Gal staining in animals carrying both c564-GAL4 and the UAS-PGRP-LC transgene was greater in infected than in uninfected animals.

Fig. 4. Inactivation of PGRP-LC by transfection of dsRNA blocks induction of antibacterial gene expression in mbn-2 cells. Northern blot detection of Dipterericin, Cecropin A1, and Attacin A in mbn-2 cells is shown after treatment with dsRNA from PGRP-LC, PGRP-LA, or lacZ and induction with the indicated elicitors. Ethidium bromide staining of ribosomal RNA was used as a loading control. mbn-2 cells were plated at a density of 1 million cells/ml and transfected 1 day later with 10 μg of dsRNA (31). For PGRP-LA the dsRNA corresponded to 935 bp from exons 2 to 5; for PGRP-LC the dsRNA corresponded to 861 bp from the common exons 2 and 3. Three days after transfection, the cells were induced with insoluble peptidoglycan from Micrococcus luteus for 6 hours, live E. coli (O55:BS) for 2 hours, or sterile Ringer (-) as control. The pellet of an E. coli overnight culture was resuspended 1:100 in sterile Ringer, and 15 μl were used per induction. Peptidoglycan and LPS had a final concentration of 1 μg/ml. The cells were harvested after 2 or 6 hours, and total RNA was extracted. The loss of PGRP-LA and PGRP-LC mRNA due to RNAi was confirmed by reverse transcription PCR in a separate experiment. Drosomycin expression is not inducible in this mbn-2 cell line, so the effect of PGRP-LC RNAi on its expression could not be assessed in this experiment.

plasmic and transmembrane domains but different extracellular domains. The extracellular PGRP domains of the two isoforms were only 38% identical (55 of 145 residues). Northern hybridization with a common PGRP-LC exon probe revealed transcripts about 2.0 kb in size in wild-type larvae, but no transcript of that size in ird7 mutations; instead, a larger transcript of lower abundance was detected (Fig. 2B). Sequence analysis revealed an insertion of 838 base pairs (bp) of single-copy sequence into exon 2, which is the first coding exon in both isoforms, in the ird7 allele (Fig. 2C). This insertion introduced a stop codon and would generate a truncated cytoplasmic protein. No sequence change in the PGRP-LCa isoform was identified in the ird7 allele. However, there was a G to A substitution in the x PGRP domain in the PGRP-LCx isoform of ird7, which introduced a stop codon that makes a truncated protein lacking the last 107 amino acids of this isoform (Fig. 2C). Because the ird7 allele alters only PGRP-LCx and has a profound effect on antimicrobial gene expression, this isoform must play a crucial role in vivo. The specific requirement for the PGRP-LCx isoform could be due to its ability to bind specific ligands or because its expression is limited to specific cell types by regulated RNA splicing. Overexpression of either of the PGRP-LCx cDNAs rescued inducible expression of the Dipterericin-lacZ reporter gene in homozygous ird7 mutant animals (Fig. 3), confirming that the phenotype of ird7 mutants was the result of the lack of PGRP-LC activity.

We used RNA interference (RNAi) to test the role of PGRP-LC in the response to bacterial components. Treatment of blood cells from the mbn-2 line with peptidoglycan, Escherichia coli, or lipopolysaccharide (LPS) led to a robust induction of the antibacterial peptide genes. Introduction of double-stranded RNA (dsRNA) of PGRP-LC, but not PGRP-LA, effectively blocked induction of
Diptericin, Cecropin A1, and Attacin A in response to all three stimuli (Fig. 4). Thus, PGRP-LC is required for the response to both peptidoglycan and LPS in these cells.

Because PGRP-LC is predicted to encode a transmembrane protein with an extracellular PGRP domain, PGRP-LC may act as a pattern recognition receptor that links recognition of microbial components with host immune responses (22). Because PGRP-LC is required for responses to both peptidoglycan and LPS, the extracellular domain of PGRP-LC may bind both peptidoglycan and LPS, and binding of either ligand may activate downstream signaling events. Alternatively, PGRP-LC may bind peptidoglycan (but not LPS) and may act as an essential subunit of a larger complex that includes other pattern recognition receptors that bind LPS. In mammals, signaling by Toll-like receptor 2 (TLR2) is activated by peptidoglycan (23). PGRP-LC might act in a complex with another transmembrane protein similar to TLR2.

Twelve PGRP genes have been identified in the Drosophila genome (18). Another Drosophila gene, PGRP-S4, encodes a soluble peptidoglycan recognition protein that is essential for activation of the Toll signaling pathway in response to infection by Gram-positive bacteria (21). Four PGRP genes have already been identified in the human genome (24). Given the evolutionary conservation of many proteins required for innate immune responses, it will be important to evaluate whether PGRPs function as a family of pattern recognition receptors in human innate immune responses.

References and Notes
17. See supplemental material on Science Online at www.sciencemag.org/cgi/content/full/1070216/DC1.
25. K.-M. Choe, K. V. Anderson, data not shown.
32. We thank R. Artero and P. Morcillo for technical advice; B. Lemaitre for Drosophila and bacterial stocks; P. J. Lewis for bacterial stocks; D. Ferrandon, N. Perrimon, and the Drosophila Stock Center for Drosophila stocks; and T. Bestor for helpful comments on the manuscript. Supported by grants from the NIH and the Lita Annenberg Hazen Foundation (K.V.A.) and from the Göran Gustafsson Foundation for Scientific Research, the Swedish Natural Science Research Council, and the Swedish Medical Research Council (D.H.) and the Swedish Natural Science Research Council (S.S.).

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