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Cactus-Independent Nuclear Translocation of *Drosophila* RELISH

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Abstract Insects can effectively and rapidly clear microbial infections by a variety of innate immune responses including the production of antimicrobial peptides. Induction of these antimicrobial peptides in *Drosophila* has been well established to involve NF-κB elements. We present evidence here for a molecular mechanism of Lipopolysaccharide (LPS)-induced signaling involving *Drosophila* NF-κB, RELISH, in *Drosophila* S2 cells. We demonstrate that LPS induces a rapid processing event within the RELISH protein releasing the C-terminal ankyrin-repeats from the N-terminal Rel homology domain (RHD). Examination of the cellular localization of RELISH reveals that the timing of this processing coincides with the nuclear translocation of the RHD and the retention of the ankyrin-repeats within the cytoplasm. Both the processing and the nuclear translocation immediately precede the expression of antibacterial peptide genes *cecropin A1*, *attacin*, and *diptericin*. Over-expression of the RHD but not full-length RELISH results in an increase in the promoter activity of the *cecropin A1* gene in the absence of LPS. Furthermore, the LPS-induced expression of these antibacterial peptides is greatly reduced when RELISH expression is depleted via RNA-mediated interference. In addition, loss of cactus expression via RNAi revealed that RELISH activation and nuclear translocation is not dependent on the presence of cactus. Taken together, these results suggest that this signaling mechanism involving the processing of RELISH followed by nuclear translocation of the RHD is central to the induction of at least part of the antimicrobial response in *Drosophila*, and is largely independent of cactus regulation. J. Cell. Biochem. 82: 22–37, 2001.

Key words: NF-κB; I-κB; cactus; antimicrobial peptides; innate immunity; RNA interference (RNAi)

The innate immune system of flies is highly adapted to promote survival through a variety of mechanisms including the rapid production of antimicrobial peptides which have antibacterial and antifungal activities [Hultmark, 1993; Hoffmann et al., 1996; Hoffmann and Reichhart, 1997]. Increasingly, it is being appreciated that many features of innate immunity are highly conserved between insects and mammals. This includes the cell-mediated surveillance functions carried out by macrophages and neutrophils in mammals and by macrophages-like cells (hemocytes) in *Drosophila* [Abrams et al., 1992; Hultmark, 1993; Rizki and Rizki, 1994; Hoffmann et al., 1996; Hoffmann and Reichhart, 1997]. In addition, the molecular mechanisms by which defense functions are mediated appear to involve conserved signaling components including those belonging to the Toll receptor pathway which were initially identified to mediate dorsal–ventral patterning in *Drosophila* development. The cytoplasmic portion of Toll closely resembles that of the interleukin-1 receptor and a family of Toll-like receptors (TLR) in mammals [Gay and Keith, 1991; Medzhitov et al., 1997]. Mutations in this pathway also appear to disrupt the antifungal response in flies implicating an innate immune function [Lemaitre et al., 1996; Tauszig et al., 2000]. And more recently, several new Toll family members have been identified in *Drosophila* suggesting that recognition of microorganisms and regulation of immune responses is more complex than first thought [Tauszig et al., 2000].

The NF-κB related factors (Rel proteins) dorsal, dorsal-related immune factor (dif), and RELISH have been implicated as active transcriptional mediators in both the antifungal

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and antibacterial responses in *Drosophila* [Engström et al., 1993; Petersen et al., 1995; Dushay et al., 1996; Gross et al., 1996]. Of these, dorsal and dif have been shown to reside within the Toll receptor pathway with dif being implicated only in the immune response [Meng et al., 1999] and dorsal having a dual function regulating both dorsoventral polarity and antimicrobial responses [Kylsten et al., 1990; Reichhart et al., 1993; Lemaitre et al., 1995; Belvin and Anderson, 1996]. Dorsal function alone is not essential for the innate immune response since dorsal mutants retain LPS inducibility [Reichhart et al., 1993], however, dif and dorsal together appear to be required for the induction of a subset of antimicrobial genes as demonstrated by dif/dorsal deletion mutants [Meng et al., 1999]. Recently, the multifaceted nature of the antimicrobial response involving Rel proteins has been further characterized and shown to resemble that of the mammalian pathway in which NF-κB components combine to form homo- and heterodimers in order to elicit specific developmental and immune functions [Yang et al., 1997; Ghosh et al., 1998; Han and Ip, 1999]. Furthermore, the I-κB orthologue, cactus, has been shown to regulate the activation of dorsal via a cytoplasmic retention mechanism analogous to that of I-κB function in mammals [Whalen and Steward, 1993; Nicolas et al., 1998].

The third NF-κB family member, RELISH, is distinct from that of dif and dorsal in its overall structure [Dushay et al., 1996; Hoffmann and Reichhart, 1997]. In addition to the RHD, RELISH also contains an I-κB-like ankyrin repeat domain similar to that found in mammalian NF-κB molecules, p105 and p100 [Dushay et al., 1996]. The presence of these dual components suggests a regulatory mechanism similar to that found in p105 and p100 where they can exist as both intact molecules and molecules with the ankyrin-repeats proteolyticly removed from the RHD [Baldwin, 1996; Ghosh et al., 1998]. While expression data indicates that the RELISH RHD can act independently or in combination with dif and dorsal to elicit antimicrobial peptide synthesis, the actual cleavage and release mechanism has not been characterized [Dushay et al., 1996; Han and Ip, 1999]. With regard to function, the central role of RELISH has recently been demonstrated using RELISH deletion mutants in whole flies. These flies exhibit dramatically increased susceptibility to bacterial and fungal infection and thereby underscore the biological importance of RELISH [Hedengren et al., 1999].

In this paper, we set out to examine the molecular nature of LPS-induced RELISH activation using *Drosophila* S2 cells as a model. *Drosophila* S2 cells show several hemocyte-like characteristics, carry out receptor-mediated endocytosis through a novel class of scavenger receptor, and secrete antimicrobial peptides in response to LPS treatment [Kylsten et al., 1990; Samakovlis et al., 1990; Abrams et al., 1992; Pearson et al., 1995; Kirkpatrick et al., 1995]. In addition, the S2 cells have been previously used to describe the nature of *Drosophila* NF-κB interactions that result in antimicrobial peptide production [Han and Ip, 1999]. We find that RELISH is rapidly processed in response to LPS stimulation releasing the N-terminal RHD from the C-terminal ankyrin-repeats. The appearance of these two species from the original RELISH molecule temporally coincides with the nuclear translocation of the RHD and the retention of the ankyrin-repeats in the cytoplasm. We have also utilized RNA interference (RNAi) technology to simulate a loss-of-function phenotype in S2 cells by blocking the expression of endogenous NF-κB and I-κB family members. The depletion of RELISH in S2 cells by this method results in a significantly reduced capacity to produce the antibacterial peptides cecropin A1, attacin, and diptericin in response to LPS stimulation. Unexpectedly, the depletion of the I-κB homolog, cactus, via RNAi did not result in enhanced expression of these antibacterial peptides but only of the antifungal peptide, drosomycin. Our results suggest that the rapid recruitment of antimicrobial peptides directly correlates with the transient processing of RELISH. Furthermore, the activation and nuclear translocation of RELISH via LPS appears to be independent of the presence of cactus since depletion of cactus expression via RNAi does not alter the cellular localization of RELISH. Taken together, our results suggest that cactus is not involved in the regulation of RELISH activation, at least not in the context of the antibacterial peptides examined here.

MATERIALS AND METHODS

Cells and Reagents

Drosophila S2 cells were cultured weekly in M3 medium (Gibco/BRL) containing 10% fetal

calf serum (Hyclone) at a density of 2×10^6 cells/ml. Gamma-irradiated LPS from *E. coli* strain 0111:B4 was purchased from Sigma. Expressed Sequence Tags (ESTs) were purchased from Genome Systems. The pMT-V5/6His vector (Invitrogen) contains a *Drosophila* metallothionine promoter with C-terminal V5 epitope and 6X His tags. The pFR-Luc vector (Stratagene) contains the firefly luciferase gene under the control of the 5XGal4 promoter. Cellfectin transfection reagent was purchased from Gibco/BRL.

Cloning and Tagging of *Drosophila* Genes

The Cecropin A1 promoter (−778 to −89 bp) was Polymerase Chain Reaction (PCR) amplified from genomic DNA using primers (5′-3′) AATAATAAGCTTAGTGGGAATTGGCTTTGC TAA and AATAATACCCGGGCGAGAGCTTT-TATAGGC and digested with *Hind*III and *Xma*I. The 5XGal4 binding site of pFR-Luc was removed by restriction digestion with *Hind*III and *Xma*I and replaced with either the Cecropin A1 promoter (CecA1-Luc) or annealed oligos containing a TATA box (Tata-Luc) only (5′-3′) (AGCTTCGCCTATAAAAGCTCTC-GCC and CCGGGGCGAGAGCTTTTATAGGC-GA). The NF-κB site within the CecA1-Luc construct was then changed (Mut-CecA1-Luc) by QuickChange site-directed mutagenesis (Stratagene) using the following primer pairs (5′-3′): GTACTTTTCTCTGCAAAAATCTTCG-TGCATGCCTTATCTGTC and GACAGATAA-GGCATGCACGAAGAT TTTTGCAGAGAAAA-GTAC. *Drosophila RELISH* was subcloned into the pMT-V5/6His vector between sites *Eco*RI and *Apa*I in a 3 part ligation with an *Apa*I/*Pin*AI 3 fragment from EST LD09609 and an *Eco*RI/*Pin*AI fragment from the PCR-amplified 5′ region of *RELISH* (primers ACGGACTA-TGAACATGAATCAG and CTTTCGGTACA-AGAGCGAGA (pRELISH)). The pRELISH construct was further modified by removal of the 3′ region of RELISH (the ankyrin repeat region) between restriction sites *Xho*I and *Xba*I leaving the RHD intact. The resultant vector (pRHD) was ligated with oligo linkers TCGAGAAGCTTT and CTAGAAAGCTTC. *Drosophila dif* was PCR amplified from poly A + RNA in two parts using primers TTA-CACGTGTCAACACAACA AAGAGCTAATTC-GATTTCTGTCTG, TCGTCGCTCCATCCGAG-GGTCTCACTAGTTTCAGC (5′ region) and AGCTTTTAAGACGCCGCGCTACAGGAACA-CCGAGAT, CAGGCTTTCTGTTGAAGTTCA-TTTTGGATTGAGACCTTTTTACA (3′ region) and the products were cloned into TA vector 2.1 (Invitrogen). The PCR product of the 5′ region of *dif* was cut out with *Eco*RI and *Dra*III and the PCR product of the 3′ region of *dif* was cut out with *Dra*III and *Xho*I. These fragments were ligated together into the pMT-V5/6His vector between *Eco*RI and *Xho*I sites (pDif). *Drosophila cactus* (Gen Bank Accession L03367) was subcloned from EST LD10910 into the pMTal vector [Angelichio et al., 1991] between *Sst*II and *Spe*I sites (pCactus). A V5 epitope tag was added on the 3′ end of the genes subcloned into the pMT-V5/6XHis vector either by site-directed mutagenesis to remove the stop codon or by oligo-linker insertion. A FLAG tag was also added to the 5′ end of the *RELISH* gene resulting in the vector pFLAG/RELISH/V5.

Luciferase Reporter Analysis

As an internal control for the transient transfection, a vector (pTα1[157/t696] lacC-P20)[O'Donnell et al., 1994] containing the LacZ gene under the control of the *Drosophila* α-tubulin promoter was co-transfected at 10% of the total reporter DNA concentration. S2 cells (1×10^5/well) were plated in 96-well plates and cultured overnight. S2 cells were transiently transfected on the following day with 750 ng/well of reporter vector with or without 500 ng/well of vector containing the appropriate *Drosophila* gene. One day later, the cultures were either stimulated with LPS for various times or with copper sulfate (500 μM) to induce gene expression. LPS stimulation was performed 24 h after copper sulfate addition. Cells were lysed with 1X luciferase cell culture lysis reagent (Promega) and one third of the lysate was analyzed for luciferase activity with luciferase assay reagent (Promega) and one third of the lysate was analyzed with Galacto-Star β-gal reagent (Tropix). Light emission was quantitated with a Packard Top Count-HTS microplate scintillation & luminescence counter. A ratio of the averages of luciferase to β-gal were used to calculate the relative fold increases. All conditions were assayed in quadruplicate.

Taqman Analysis (Quantitative PCR)

Primer pairs and fluorescently labeled probes were designed to the *Drosophila* genes *actin 5C*, *cecropin A1*, *attacin*, *diptericin*, and *drosomycin* and purchased from PE Applied Biosystems.

The primer pairs used were (5′-3′) TTGCTGCTCTGGTTGTCGAC and CATCGTCTCCGGCAAATCC (*actin 5C*), GTTGGTCAGCACACTCG and ATTGGCGGCTTGTTGAGC (*cecropin A1*), AATCCCAACCACAATGTGGT and ACCGGACCGCTTTGAGTG (*attacin*), TGGCTTTGCAGTCCAGGG and TCCAATCTCGTGGCGTCC (*diptericin*), ATGCTGGTGGTCCTGGGA and CTTGTATCTTCCGGACAGGCA (*drosomycin*). The fluorescently labeled probes used were 6FAM-ACGGCTCTGGCATGTGCAAGGC-TAMRA (*actin 5C*),6FAM-TTCCCAGTCCCTGGATTGTGGCA-TAMRA (*cecropin A1*), 6 FAM-TCAGGTTTTCGCCGCCGGAA-TAMRA (*attacin*), 6FAM-TTGTCGCTGGTCCACACCTTCTGGA-TAMRA (*diptericin*), 6FAM-CCAACGAGGCCGATGCCGA-TAMRA (*drosomycin*). Total RNA (5 μg) was reverse transcribed using Superscript Preamplification kit (Gibco/BRL). Taqman analysis was performed on an ABI Prism 7700 sequence detection system using the manufacturer recommended cycling conditions. A standard curve of copy numbers of S2 cell genomic DNA (ranging from 25 to 2.5×10^6 copies) was used to quantitate the message levels present in the reverse-transcribed samples. A 1:500 dilution of the reverse-transcribed samples was found to be optimally within the linear range of the standard curves for the genes analyzed. All samples were analyzed in triplicate and the copies of message detected were normalized relative to the actin 5C values. From the normalized data, relative fold increases were determined and statistical significance between groups was calculated using a two-tailed t test.

RNAi Preparation and Analysis

Primers were tailed with either T3 or T7 promoter sequences for transcription of sense and antisense RNA, respectively. PCR primers were designed to *RELISH* (5′-3′) (ATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAGTACTACGACCTGGACAATG and GACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGAGCAACGCCGAAACTAACG; corresponding to nucleotide 13 to 315 of the coding sequence), *dif* (ATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGTTACACGTGTCAACACAACAAAGAGCTAATTCGATTTCTGTCTG and GACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGTCGTCGCTCCATCCGAGGGTCTCACTAGTTTCAGC; corresponding to nucleotide −138 of untranslated region to 1042 of the gene), *dorsal* (ATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGGGGCAGGGTCCAGCGGTTGAT and GACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGGGGCGGCAGTGGCGAGGTGA; corresponding to nucleotide 249 to 844 of the coding region), and *cactus* (ATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAAAGCAGCGGAGGCAGCAACAAAG and GACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCCACGTCCACTGATCCCGAAATAC; corresponding to nucleotide 213–934 of the coding region) and were used to amplify the fragments of these genes. It should be noted that the region of the cactus gene amplified and used for RNAi is identical in both the maternal and zygotic forms of cactus. The riboprobe *in vitro* transcription kit (Promega) was used to synthesize sense and antisense RNA from the PCR products (large-scale preparation). Following DNase treatment, RNA transcript was phenol extracted, ethanol precipitated, and resuspended in 5 mM Tris pH 7.9. Duplexed RNA was generated by heating equal amounts of sense and antisense RNA to 80°C for 5 min in a tube immersed in 150 ml water and allowing to cool to room temperature for 1 to 2 h. Sense, antisense, or duplexed RNA were transiently transfected (7.5 μg/5×10^6 cells) with cellfectin into the S2 cells. Since double-stranded RNA migrates more slowly than single-stranded RNA by agarose gel electrophoresis, preparations of duplexed RNA were confirmed by native agarose gel electrophoresis to migrate more slowly.

Western Blot Analysis

S2 cells (1×10^7) were harvested and washed in ice cold PBS three times by centrifugation for 10 min at 300*g*. Pelleted cells were lysed on ice for 20 min in 50 mM Tris pH 8.0, 150 mM sodium chloride, 1% IGEPAL, and 2 mM EDTA. Lysates were centrifuged 10 min at 14,000*g* and the soluble fraction was combined with sample buffer containing 10 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.2% bromophenol blue and boiled for 5 min. Samples were loaded onto a precast 10% acrylamide gel (NOVEX) and electrophoresed. The acrylamide gel was transferred to Protran nitrocellulose (Schleicher & Scheull), blocked overnight with 5% milk in Tris-buffered saline plus 0.5% Tween-20 (TBS-T). The blot was incubated with anti-V5 epitope antibody (Invitrogen) or

anti-FLAG tag M2 antibody (Stratagene) in 5% milk-TBS-T for 1 h at RT before being washed 4 times (15 min each) with TBS-T. The blot was then incubated for 1 h at RT in 5% milk-TBS-T plus secondary antibody (HRP-labeled; Jackson Immuno-Research) and again followed by washing with TBS-T 4 times. The blot was developed with Western Blot Chemiluminescence reagent (NEN Dupont) and exposed to x-ray film.

Immunocytochemistry

S2 cells were cultured in 6-well plates at a density of 3×10^6 cells/well and transfected with 15 μg of pRELISH/FLAG/V5 vector. Gene expression was induced by copper sulfate for 24 h. Cells were transferred to poly-lysine coated LabTek II microculture slides and cultured overnight. Following LPS stimulation, the cells were fixed with 1% paraformaldehyde in PBS for 30 min and washed twice with PBS. Cells were permeablized with PBS containing 5% goat serum (Sigma) and 0.1% Triton X-100 for 1 h. Primary antibody was added at 1 μg/well in PBS/5% goat serum for 1 h and washed six times with PBS/5% goat serum. Secondary antibody (Cy2 labeled goat anti-mouse IgG (Jackson Immunoresearch)) was added at 1 μg/well in PBS/5% goat serum for 1 h and washed six times with PBS/5% goat serum. DAPI was added for 15 min at 0.1 μg/well and washed twice with PBS/5% goat serum. The slides were allowed to air dry before mounting with ProLong Antifade (Molecular Probes). Images were visualized with an Olympus BX60 microscope and Hamamatsu color chilled 3 CCD camera unit.

RESULTS

Drosophila S2 Cells Respond to LPS Stimulation

Based upon recent reports citing the LPS responsiveness of *Drosophila melanogaster* cell lines [Georgel et al., 1995; Petersen et al., 1995; Dushay et al., 1996; Gross et al., 1996; Han and Ip, 1999], we set out to characterize this response in S2 cells and to use the unique properties of this system to model signal transduction events. We developed a luciferase reporter assay similar to that as described by Engström et al. [1993] to assess the achievable antimicrobial response as measured through cecropin A1 promoter activity. We found that LPS could dose-dependently activate the cecropin A1 promoter over a wide concentration range from 1 μg/ml to 1 mg/ml with a half-maximum value of ~100 μg/ml (Fig. 1). In contrast, LPS failed to induce luciferase production in S2 cells that were transfected with a reporter vector containing an inactive NF-κB site (Mut-CecA1-Luc) or a minimal promoter (Tata-Luc) (Fig. 1). Based on these kinetics, we chose a concentration (50 μg/ml) just below the half-of maximal value. These data demonstrate that LPS induces a robust NF-κB-specific antimicrobial peptide response.

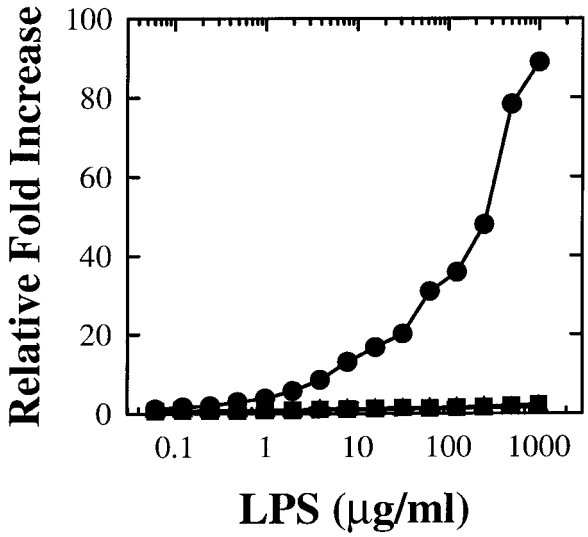

Fig. 1. LPS dose-dependent cecropin A1 promoter activation in S2 cells. S2 cells were transiently transfected with luciferase reporter vectors CecA1-Luc (●), Mut-CecA1-Luc (■), or Tata-Luc (▲) followed by stimulation with various concentrations of LPS. Forty-eight hours later, the S2 cells were lysed and analyzed for luciferase and β-gal activity. Luciferase activity was normalized to β-gal activity and these ratios used to determine the relative-fold-increase of luciferase activity between groups. The relative fold increase values are in relation to transfected non-stimulated S2 cells. All groups were assayed in quadruplicate. Standard deviation ranged from 5 to 20% of the mean. These results are representative of three independent experiments.

To more precisely define the timing of this LPS response, we followed the mRNA production of multiple antimicrobial peptide genes following LPS stimulation. Primers and probes were designed for Taqman (Quantitative RT-PCR) analysis of the antibacterial peptides cecropin A1, attacin, and diptericin and the antifungal peptide, drosomycin. Standard curves were generated for all analyzed genes using S2 cell genomic DNA. *Drosophila actin 5C* transcripts calculated by this method were used for normalization of the antimicrobial peptide

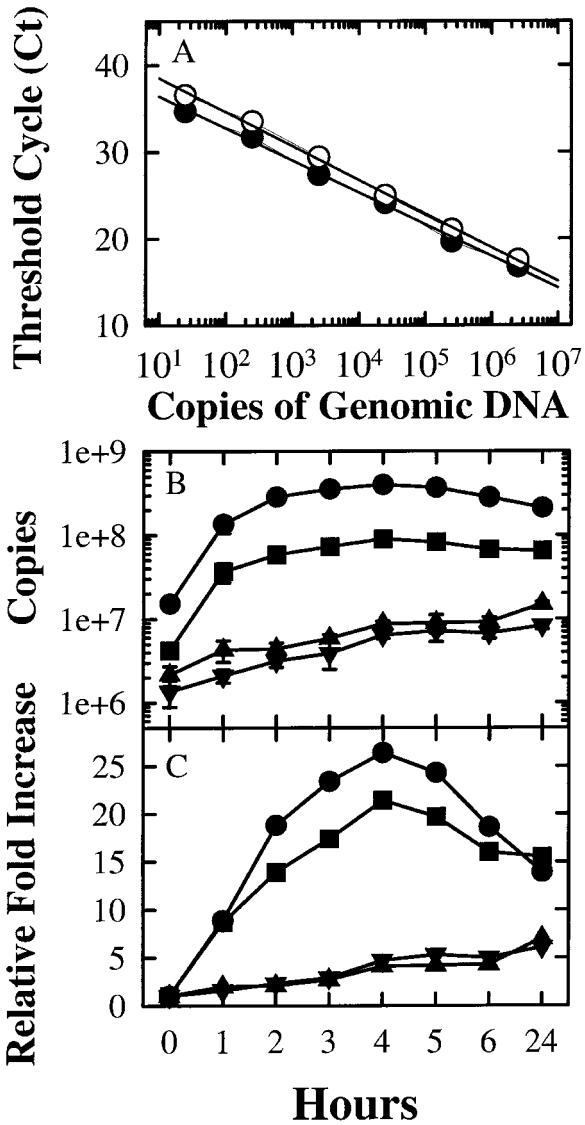

Fig. 2. LPS time-dependent induction of cecropin A1, attacin, diptericin, and drosomycin mRNA. S2 cells were stimulated with LPS (50 μg/ml) for various times. Transcription of individual genes was assessed by Taqman (Quantitative RT-PCR) analysis. **A:** Defined copies of genomic DNA from S2 cells were used to determine standard curves for actin5C (○) and cecropin A1 (●). Standard curves for attacin, diptericin, and drosomycin were similar to actin5C and cecropin A1 (data not shown). Least-squares analysis was used to calculate a standard curve for each gene analyzed. **B:** Mean values of cecropin A1 (●), attacin (■), diptericin (▲), and drosomycin (▼) mRNA copies in S2 cells following LPS for various times are shown. Data was normalized using mean actin5C values for each group. All groups were assayed in triplicate. **C:** Relative fold increase in mRNA was determined from non-treated S2 cells. These results are representative of three independent experiments.

transcripts. Representative standard curves for *actin 5C* and *cecropin A1* are shown in Figure 2A. Taqman analysis of S2 cell mRNA revealed an LPS-induced, time-dependent increase of both cecropin A1 and attacin transcripts reaching a maximum by 4 h followed by a steady decrease to 24 h (Fig. 2B and C). However, both diptericin and drosomycin were poorly expressed. Low diptericin expression was also confirmed via luciferase reporter using the diptericin promoter (data not shown). Importantly however, S2 cells clearly possess the signaling components necessary for LPS-induced production of the antimicrobial peptides, cecropin A1 and attacin.

LPS Induces RELISH Protein Processing

Since RELISH has been shown to be central to the induction of these antimicrobial peptides and the overall structure of RELISH is similar to that of mammalian NF-κB, p105, we were interested in examining the nature of RELISH activation [Dushay et al., 1996; Hedengren et al., 1999]. Intact full-length RELISH modified with an N-terminal FLAG tag and a C-terminal V5 epitope tag was transiently expressed in S2 cells. RELISH expression was detected by Western analysis using either anti-FLAG or anti-V5 antibody (Fig. 3). We observed in several independent experiments that the efficiency of RELISH processing varied. Nevertheless, the LPS stimulation of S2 cells overexpressing RELISH consistently resulted in an extremely rapid processing event converting the intact RELISH molecule (110 kDa) to an approximate 62 kDa (anti-FLAG) and 52 kDa species (anti-V5) within 1 h of induction (Fig. 3; lanes 1 and 2 each). As a control, the zygotic I-κB *Drosophila* orthologue, cactus, was expressed and analyzed under similar conditions as RELISH. Unexpectedly, and in contrast to RELISH, LPS did not appear to induce degradation of cactus (Fig. 3; lanes 7 to 12). These data suggest that RELISH can participate in LPS signaling via a proteolytic processing mechanism releasing the ankyrin-repeat element from the RHD.

We were also interested in identifying the site of cleavage within the RELISH protein. Mammalian p105 and p100 molecules contain a glycine-rich region that acts as a hinge exposing the protein for proteolytic processing [Lin and Ghosh, 1996]. Likewise, RELISH contains a serine-rich region that is believed to act in the

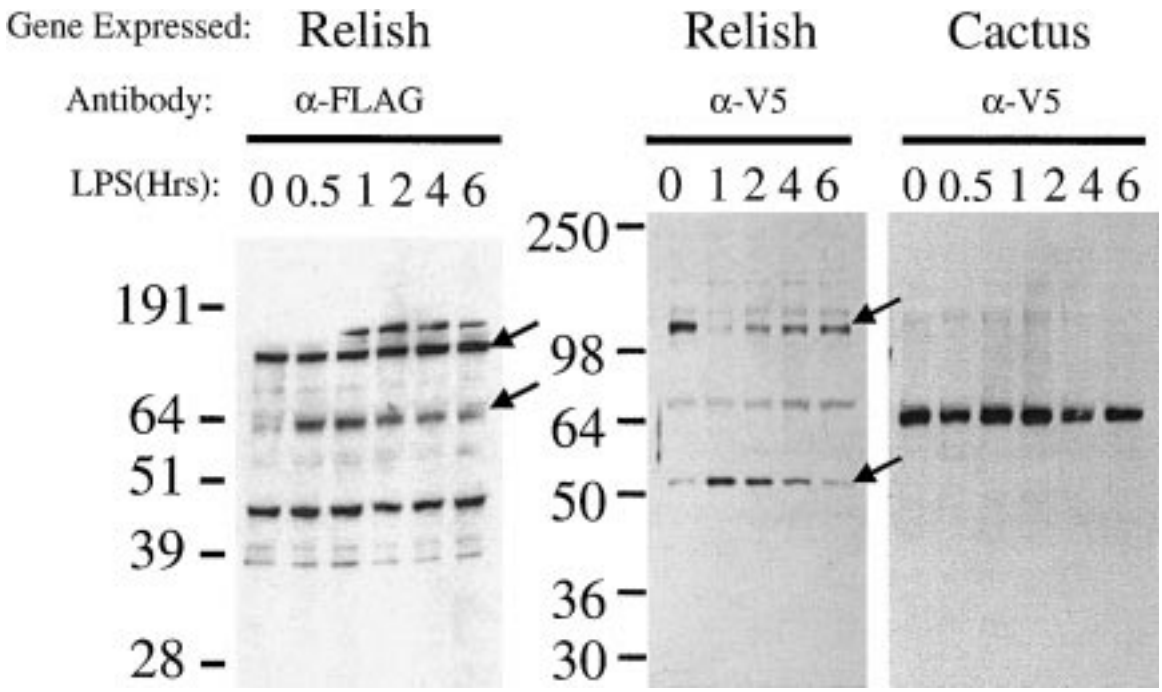

Fig. 3. LPS-induced processing of RELISH. S2 cells were transiently transfected with either pFLAG/RELISH/V5 or pCactus/V5 and stimulated with or without LPS (50 μg/ml) for various times. S2 cells were lysed and equivalent amounts of lysate were analyzed by Western blotting using either anti-FLAG antibody or anti-V5 antibody. Arrows indicate RELISH protein. These results are representative of three independent experiments which varied in the efficiency of processing.

same capacity [Dushay et al., 1996]. We decided to mutate or delete this serine-rich region in an effort to block the processing event induced by LPS. Consistent with studies of the glycine-rich region of p105 [Lin and Ghosh, 1996], alterations to the serine-rich region failed to block this processing event (data not shown). Thus, the cleavage site remains undefined. However, based on the size of the two species observed following LPS stimulation, we estimate that the cleavage site is just C-terminal to the serine-rich region.

Over-Expression of RHD Stimulates Cecropin A1 Promoter Activity

To further investigate the nature of endogenous RELISH, we analyzed cecropin A1 promoter activity via luciferase reporter in cells expressing either intact RELISH or the RHD of RELISH in the presence or absence of LPS stimulation. Only over-expression of RHD increased the basal levels of luciferase activity (to the same level as induced by LPS; 6.4-fold) in the absence of LPS stimulation suggesting that the ankyrin-repeats were necessary for maintaining RELISH in an inactive form (Fig. 4). Furthermore, over-expression of cactus did not inhibit the LPS-induced cecropin A1 promoter activity (Fig. 4). Our results are consistent with those reported by Dushay et al. [1996] and provide support to the observation in Figure 3 that the full-length RELISH molecule appears to reside as a full-length intact molecule in *Drosophila* cells until LPS is encountered.

LPS-Induced Nuclear Translocation of RELISH

The observation that RELISH is processed in response to LPS stimulation leads to the question of the ability of RELISH to translocate to the nucleus and thus be available to mediate transcription. To address this issue, S2 cells expressing RELISH with the N-terminal FLAG tag and the C-terminal V5 tag were stimulated with or without LPS for various times (Fig. 5). Immunostaining of these cells with either anti-FLAG or anti-V5 antibody demonstrated that a majority of fluorescence was localized to the cytoplasm in non-stimulated cells. Within 15 min of LPS stimulation, the fluorescence was reduced in the cytoplasm and observed to increase in the nucleus of anti-FLAG stained cells. This was in contrast to cells stained with anti-V5 antibody where the fluorescence remained in the cytoplasm. As a control, the

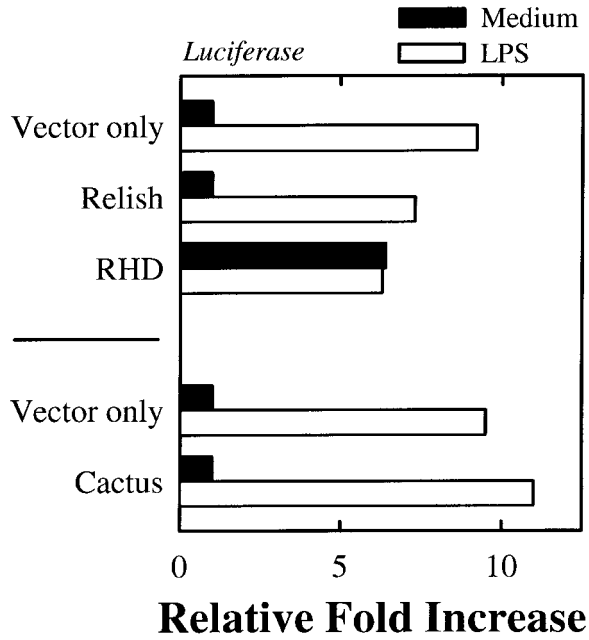

Fig. 4. Effect of RELISH over-expression on Cecropin A1 promoter activity. S2 cells were transiently transfected with pRELISH, pRHD, or pCactus. Following induction of gene expression, these groups were stimulated with (open bars) or without (solid bars) LPS (50 μg/ml) for 48 h. As in Figure 1, mean luciferase values were normalized with mean β-gal values and reported as a relative fold increase. All groups were assayed in quadruplicate. Values are reported as relative-fold-increases compared to empty vector transfected S2 cells. All groups were assayed in triplicate. These data are representative of at least two independent experiments. Standard deviation values were within 5 to 20% of the mean.

location of the nucleus is shown by counter stain with DAPI and is represented in merged images of anti-FLAG or anti-V5 staining. Together these results demonstrate that upon LPS stimulation RELISH is processed and the N-terminal RHD species is translocated to the nucleus with retention of the C-terminal domain in the cytoplasm.

RNAi-Mediated Interference of RELISH, Dif, Dorsal, and Cactus

Recently it has been demonstrated that double-stranded RNA duplexes derived from specific cDNA sequences can be used to specifically target protein expression when injected into either *C. elegans* or *Drosophila* embryos [Kennerdell and Carthew, 1998; Montgomery et al., 1998; Misquitta and Paterson, 1999]. We have exploited this technology to study the impact of disrupting specific signaling components of S2 cells. To demonstrate the effectiveness of dsRNA on protein expression, we transiently transfected S2 cells with dsRNA derived from either RELISH or dif coding sequence. One day following transfection, the cells were re-transfected with RELISH or dif expression vectors. Western blot analysis of RELISH and dif was then performed using anti-V5 antibody to detect expression of tagged RELISH and dif proteins. The results demonstrate that in the absence of dsRNA, both RELISH and dif are expressed (Fig. 6, lanes 1 and 2). However, in S2 cells which received RELISH-specific dsRNA, RELISH protein was not detected while dif protein was expressed to comparable levels as the control (Fig. 6, lanes 3 and 4). Conversely, in S2 cells transfected with dsRNA derived from dif, dif protein expression levels were less than 10% as compared to controls. In contrast RELISH protein expression was not affected (Fig. 6, lanes 5 and 6). Even though the mechanism of RNAi is not completely understood, these results clearly demonstrate that RNAi can be used to effectively and gene-specifically target and block protein expression in *Drosophila* S2 cell culture.

We next wished to determine the capacity of S2 cells to produce the antimicrobial peptides following RNAi-mediated loss-of-expression of endogenous RELISH, dif, dorsal, or cactus. As expected, S2 cells depleted of RELISH expression via RNAi were incapable of expressing significant levels of cecropin A1, attacin, or diptericin mRNA in response to LPS stimulation (Table I). Likewise, depletion of dorsal in S2 cells reduced but did not eliminate LPS induction of the antibacterial peptides. Meanwhile, the loss of dif resulted in a modest increase in LPS-induced antimicrobial peptide synthesis. The importance of dorsal and dif for antimicrobial responses have been addressed elsewhere [Hedengren et al., 1999]. We were surprised to find that the loss-of endogenous cactus expression via RNAi did not result in the dramatic increase in basal levels of mRNA of these antibacterial peptides above the sense control (Table I). However, as would be predicted, loss of cactus did significantly elevate the basal level of the antifungal peptide, drosomycin.

Since the loss of cactus in S2 cells did not appear to impact the LPS-induced antibacterial response, we wished to determine whether cactus was involved in LPS-induced RELISH activation. S2 cells depleted of cactus expression via RNAi were transiently transfected with an expression vector containing dif/V5 and

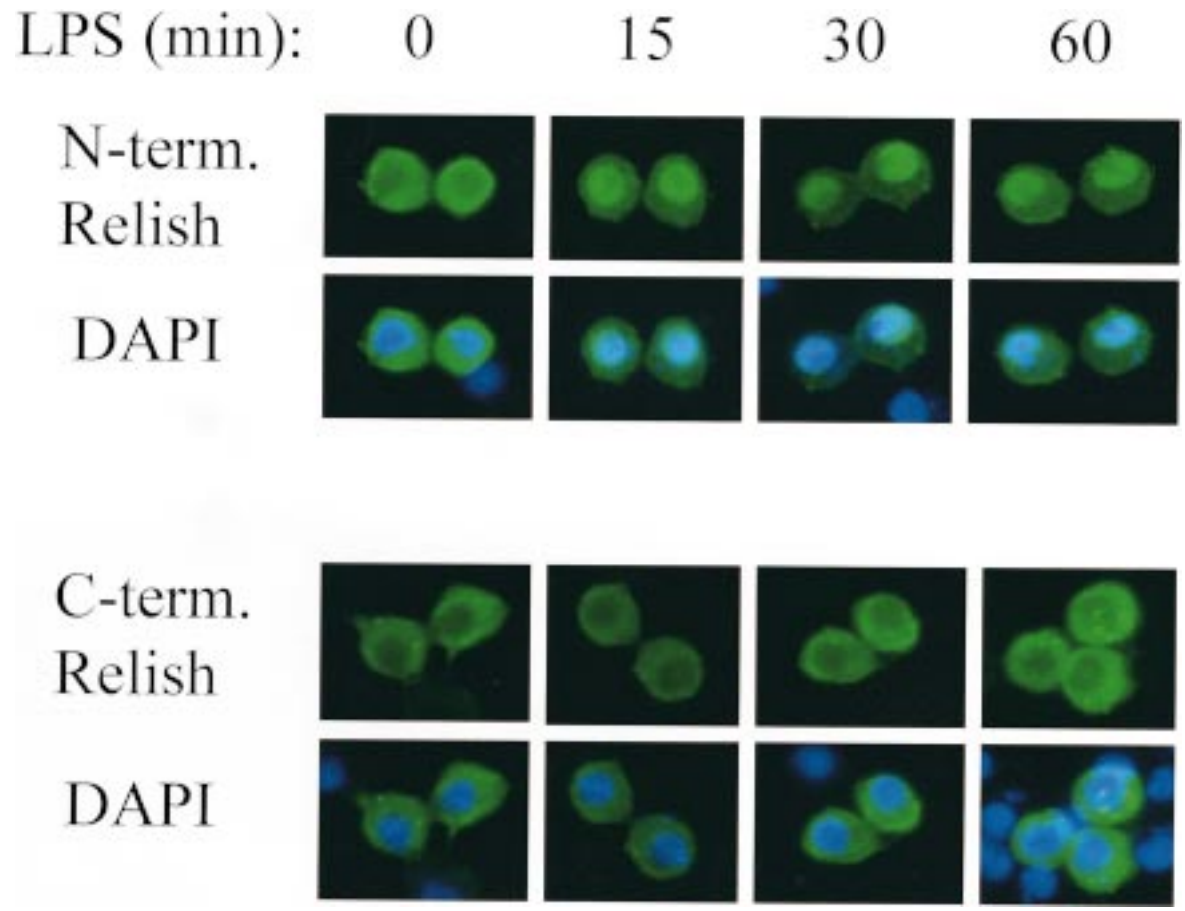

Fig. 5. Nuclear translocation of RELISH protein following LPS stimulation. S2 cells were transiently transfected with pFLAG/RELISH/V5. One day post induction of gene expression, the S2 cells were stimulated with LPS (50 μg/ml) for 0, 15, 30, or 60 min, fixed, and stained with either anti-FLAG or anti-V5 antibody. A DAPI counter stain (blue) is merged and shown directly below images to identify nuclei. All images were visualized at 600X.

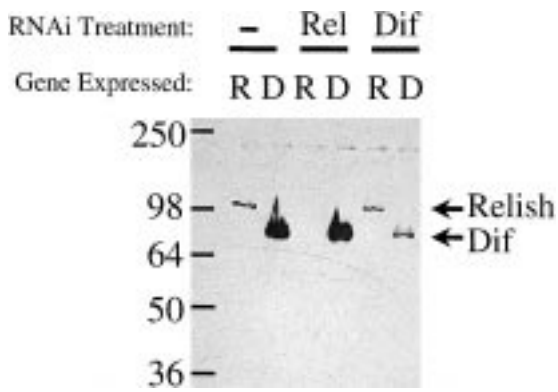

Fig. 6. RNAi-induced gene-specific modulation of RELISH and dif in S2 cells. S2 cells were transiently transfected with either nothing (**lanes 1** and **2**) or 7.5 μg/ 5×10^6 cells of dsRNA derived from *RELISH* (**lanes 3** and **4**) or *dif* (**lanes 5** and **6**). One day later, these same cells were transiently transfected with expression constructs containing either pRELISH/V5 (R) or pDif/V5 (D) genes and gene expression was induced for 24 h. Lysates were prepared from these cells and analyzed by Western blotting with anti-V5 antibody. These results are representative of two independent experiments.

examined by immunostaining. S2 cells with normal cactus expression (Fig. 7A; Mock control) were observed to express dif/V5 only in the cytoplasm. While in cactus negative cells, dif/V5 was only expressed within the nucleus (Fig. 7A; Cactus RNAi). A DAPI counter stain was used to locate the nuclei. These results demonstrate that cactus RNAi has depleted cactus protein expression and thus dif can no longer be retained in the cytoplasm.

This analysis was extended to the study of LPS-induced RELISH processing. RELISH/FLAG/V5, as in Figure 5, was expressed in S2 cells that were depleted of cactus via RNAi (Fig. 7B) and stimulated with LPS for 0, 15, and 30 min. Immunostaining of these cells demonstrates the same cellular distribution of RELISH expression as in Figure 5 when cactus was present. Full-length RELISH was expressed and retained in the cytoplasm. Upon LPS stimulation, N-terminal RELISH was translo-

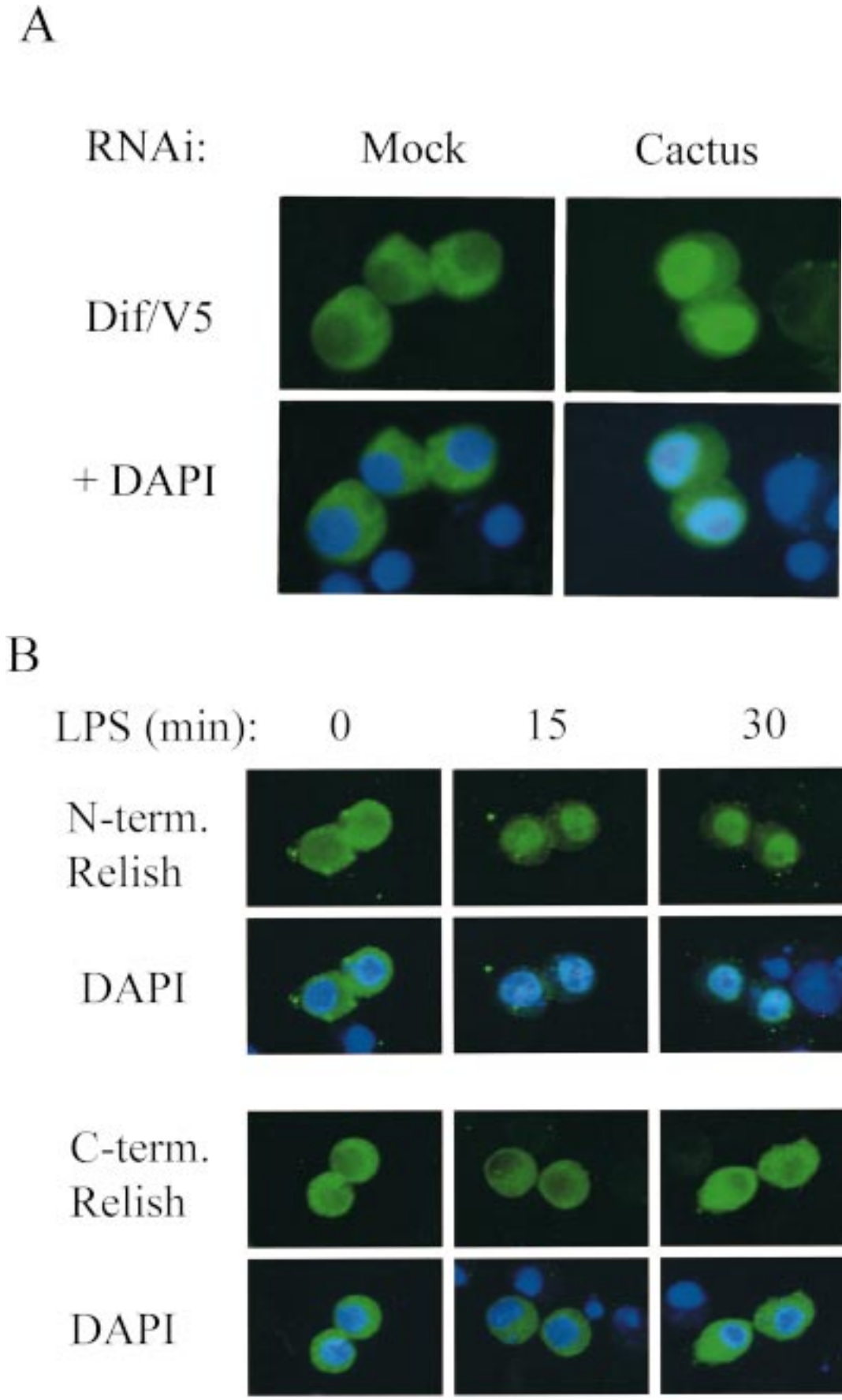
A
RNAi:
Mock
Cactus
Dif/V5
+ DAPI
B
LPS (min):
0
15
30
N-term.
Relish
DAPI
C-term.
Relish
DAPI

TABLE I. Induction of Antimicrobial Peptides Following RNAi of RELISH, Dif, Dorsal, or Cactus

	Cecropin A1	Attacin	Diptericin	Drosomycin
LPS	+	+	+	—
Control	100% (35.9)	100% (42.3)	100% (9.5)	—
RELISH sense	63.2%	99.2%	67.4%	—
RELISH duplex	6.4%****	16.5%***	11.6%***	—
Control	100% (14.3)	100% (15.7)	100% (6.8)	—
Dif antisense	85%	87.3%	94.1%	—
Dif duplex	171%**	148%****	138%****	—
Control	100% (12.9)	100% (22.4)	100% (4.7)	—
Dorsal sense	83.7%	90.2%	80.9%	—
Dorsal duplex	55.0%*	68.3%	74.5%	—
LPS	—	—	—	—
Control	100% (1)	100% (1)	100% (1)	100% (1)
Cactus sense	130%	130%	160%	280%
Cactus duplex	180%	140%	190%	880%****

Relative fold increases are shown in parentheses and were converted to percentages for means of comparison across groups with or without the LPS response of the control assigned a value of 100%. Statistical significance is indicated by underline with corresponding values of *($P < 0.05$), **($P < 0.01$), ***($P < 0.005$), and ****($P < 0.001$).

cated to the nucleus and the C-terminal ankyrin repeats remained in the cytoplasm. These results taken together demonstrate that antibacterial peptide induction in S2 cells via LPS is regulated through processing of the RELISH molecule as opposed to degradation and release of cactus from the RELISH RHD molecule.

DISCUSSION

In this report, we present evidence for the molecular mechanism of *Drosophila* RELISH activation by LPS. We have found that RELISH when expressed is an intact 110 kDa protein and LPS stimulation is required to induce a processing of this molecule into two species containing either the N-terminal RHD region or the C-terminal ankyrin-repeats. This processing is immediately followed by the rapid nuclear translocation of the N-terminal RHD which leads to the transcriptional activation of antimicrobial peptide genes, cecropin A1 and attacin. Consistent with this, over-expression of RHD alone leads to the LPS independent activation of the antimicrobial gene synthesis while over-expression of full-length RELISH in the absense of LPS has no effect. Furthermore, by taking advantage of RNAi technology we have also examined the nature of the *Drosophila* NF-κB and I-κB members in the context of this LPS stimulation. We have found that depletion of RELISH expression severely impairs the capacity of S2 cells to express several antibacterial peptides while the depletion of I-κB, cactus, did not affect the ability of the S2 cells to express these antibacterial peptides. Conversely, loss of cactus expression did not result in a significant increase in the basal levels of these antibacterial peptides. However, depletion of cactus expression did result in a significantly elevated basal level of an antifungal peptide, drosomycin. Furthermore and in contrast to dif expression analyses, loss of cactus via RNAi did not result in the automatic nuclear translocation of RELISH. Collectively these results suggest that RELISH is a central regulator of at least part of the LPS-induced antimicrobial response in *Drosophila*.

The identification and initial characterization of RELISH has been reported by Dushay et al. [1996]. They have shown that RELISH is similar in overall structure to mammalian

Fig. 7. Nuclear translocation of RELISH and dif in S2 cells depleted of cactus via RNAi. **A:** S2 cells were transfected with either nothing (Mock) or dsRNA derived from cactus. One day later, an expression vector containing dif with a V5 tag was transfected into S2 the cells and dif/V5 expression was induced with copper sulfate. Cells were fixed and immunostained with anti-V5 antibody 24 h later. **B:** S2 cells were transfected with cactus dsRNA. One day later, an expression vector containing RELISH with an N-terminal FLAG tag and a C-terminal V5 tag was transfected into S2 cells and expression was induced by copper sulfate. Twenty four hours post RELISH expression, S2 cells were stimulated with LPS for 0, 15, and 30 min followed by immunostaining with either anti-FLAG or anti-V5 antibodies. A DAPI counter stain (blue) is merged and shown directly below images to identify nuclei. All images were visualized at 600X.

NF-κB molecules, p105 and p100, containing both the RHD domain and a C-terminal ankyrin repeat domain. The p105 and p100 molecules can exist as either intact molecules or processed molecules (p50 and p52, respectively) containing only the RHD domain [Baldwin, 1996; Ghosh et al., 1998]. In addition, the processed molecules are complexed with I-κB molecules [Baldwin, 1996; Ghosh et al., 1998]. And it is this association with I-κB that regulates the activation of the processed p105 and p100 molecules. There is however, a pool of non-processed p105 present which appears to be necessary for immune responses to pathogens. In studies of mice that are null for expression of the intact p105 molecule, but do express the processed p50 form, the mice demonstrate an increased susceptibility to opportunistic infections [Ishikawa et al., 1998]. These results demonstrate that there is a population of non-processed p105 molecules that regulates and is important for aspects of immune responses independent of the I-κB regulated signaling pathways.

Another similarity between RELISH and p105 is that both contain serine-rich and glycine-rich regions, respectively, positioned between the RHD and ankyrin repeat regions. As with the glycine rich region of p105 [Lin and Ghosh, 1996], attempts to mutate or delete the serine-rich region of RELISH did not block the ability of this molecule to be processed following LPS stimulation. Our data suggests that RELISH probably exists predominately as a non-processed form in *Drosophila* in the absence of an activation signal.

In light of the processing event of RELISH, we were interested in examining the activation mechanisms associated with this event. We found that the RHD domain was rapidly translocated to the nucleus as has been shown similarly in analyses of dif and dorsal in whole flies [Lemaitre et al., 1995; Govind et al., 1996; Wu and Anderson, 1998]. Our results differ, however, in that dif and dorsal nuclear translocation has been shown in whole flies to require either the absence of cactus (i.e., in cactus -/- flies) or the degradation of cactus as a result of bacterial infection [Wu and Anderson, 1998]. Our results demonstrating that cactus was not degraded following LPS stimulation and depletion of cactus expression neither augmented the basal levels of antibacterial peptide mRNA nor induced nuclear translocation of non-processed

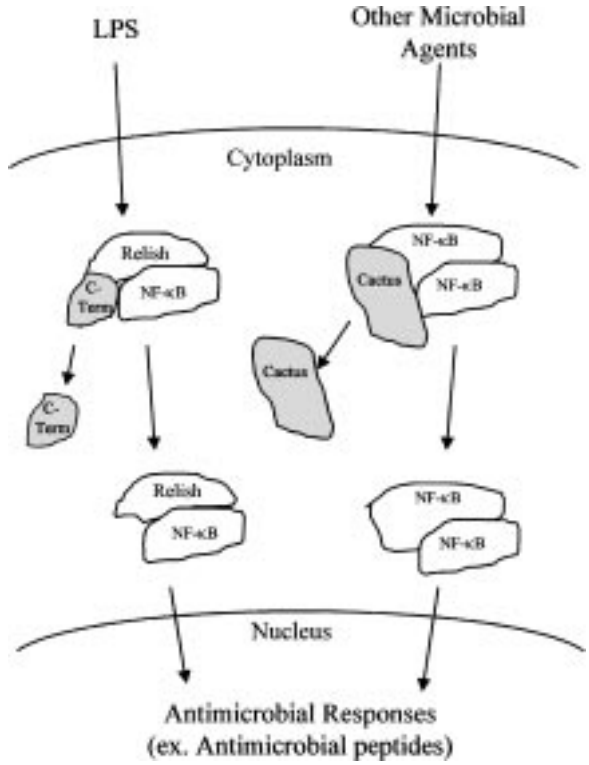

Fig. 8. Model of RELISH activation by LPS. Shown is a generalized model of RELISH activation. LPS from a microorganism in the environment induces processing of RELISH to release the C-terminal ankyrin-repeats. The RELISH complex can translocate to the nucleus to induce expression of antimicrobial peptides, cecropin A1 and attacin. Other immunostimulatory signals from microorganisms can induce cactus-mediated release of NF-κB molecules and translocation to the nucleus.

RELISH suggest that cactus is not required for LPS-induced RELISH activation. As a control for cactus RNAi, we confirmed that dif expression was localized to the cytoplasm in mock transfected cells. While in cactus depleted cells, dif was localized to the nucleus. We extended the analysis further to show that despite the depletion of cactus, expression of RELISH was localized to the cytoplasm and not to the nucleus of S2 cells until LPS stimulation was provided. This evidence strongly favors a model of LPS activation that is separate from other antimicrobial responses involving cactus (Fig. 8).

In an effort to simplify the molecular analysis of the innate immune response to bacteria, we chose to focus on LPS-induced events since it is difficult to discriminate between the effects of whole bacteria and individual immunostimulatory bacterial components. This approach is

necessary especially in light of the identification of numerous TLR in both mammals and *Drosophila* [Medzhitov et al., 1997; Rock et al., 1998; Hoshino et al., 1999; Tauszig et al., 2000]. Interestingly, some of these TLRs have been demonstrated to be specific receptors for different components of bacterial cell walls. For example, TLR4 has been clearly demonstrated to be necessary for LPS responsiveness in mammalian cells [Hoshino et al., 1999] while TLR2 has been demonstrated to be important for mediating responses to peptidoglycan and lipoteichoic acid, the predominant stimulatory components of gram-positive bacteria [Schwandner et al., 1999; Takeuchi et al., 1999]. More recently, additional TLR molecules have been identified in *Drosophila* [Tauszig et al., 2000]. Currently, two TLRs appear to be involved in antifungal responses while the other molecules appear to be involved in development. However, the presence of multiple Toll family members along with the observation that *Drosophila* antimicrobial peptide expression differs with a variety of microorganisms [Lemaitre et al., 1997] suggests that there are additional receptors that exist. Furthermore, the discrimination between the components of the bacterial cell walls from these various microorganisms is important to provide the correct immune response and thus elimination of infection.

We chose to focus on *Drosophila* S2 cells for these studies for the following reasons. First, *Drosophila* S2 cells have been previously used for studies involving biochemical analyses of innate immune responses [Han and Ip, 1999]. These studies indicate that the underlying molecular mechanisms in S2 cells reflect those found in whole flies. Second, despite being derived from a heterogenous source, S2 cells in fact are quite homogenous in nature and exhibit several proteries of hemocytes including scavenger receptor mediated endocytosis and LPS-induced expression of antimicrobial peptide genes [Kylsten et al., 1990; Samakovlis et al., 1990; Abrams et al., 1992; Pearson et al., 1995; Kirkpatrick et al., 1995]. Third, S2 cells have been shown to be very useful as a model for dissection of intracellular signaling events since the discovery of the phenomenon of RNAi [Kennerdell and Carthew, 1998; Montgomery et al., 1998; Clemens et al., 2000]. The results presented in this paper are likely to reflect the molecular events occurring in the subset of cells/tissues from which S2 cells were derived and may or may not reflect the complexity of the mechanisms occurring in whole flies. Nevertheless, S2 cells do appear to be a good model in which LPS-induced signaling responses can be studied.

LPS-induced mechanisms have been examined in *Drosophila* by several investigators and have been typically performed with relatively high doses (as compared to mammalian studies) of LPS ranging from 20 μg/ml to an average of 100 μg/ml [Samakovlis et al., 1990, 1992; Kappler et al., 1993]. In studies of hemocyte cell lines from other species such as Lepidoptera, LPS has been used at concentrations up to 1 mg/ml [Wittwer et al., 1997]. Even though these LPS concentrations are significantly higher in relation to studies with mammalian cells, these concentrations are within reasonable parameters for study in *Drosophila*. Thus, we chose 50 μg/ml based on the pharmacological kinetics of the cecropin A1 promoter activity (Fig. 1).

RNAi has been an area of intense research due to the nature of this phenomenon. It offers rapid and efficient depletion of any gene of interest and is a powerful tool for use in dissecting signal transduction pathways [Clemens et al., 2000]. It is particularly useful when deletions of your gene of interest are lethal to a developing organism. In this case preparing homozygous mutants of a gene in whole flies cannot be accomplished. However, utilizing cell lines as a model, RNAi can be an effective tool to studying these signaling components [Clemens et al., 2000].

Our analysis of RELISH using RNAi is consistent with observations made in S2 cells stably expressing the various NF-κB molecules [Han and Ip, 1999]. RNAi analysis of cactus, however, provides us additional insight to the mechanism of activation of RELISH. Our results demonstrate that depletion of cactus expression results only in an increased basal level of the antifungal peptide, drosomycin, and does not result in increased basal levels of antibacterial peptide expression. Since RELISH is important in these antibacterial responses, we conclude that cactus is probably not complexed with RELISH for the following reasons. First, based on our data, cactus is not degraded following LPS stimulation. Secondly, loss of cactus expression did not affect expression of the antibacterial peptides, only the antifungal peptide. This is consistent with reports in

the literature demonstrating that cactus is coupled to the Toll signaling pathway and regulation of antifungal responses [Lemaitre et al., 1996; Nicolas et al., 1998]. Finally, depletion of cactus does not alter the cellular expression pattern of RELISH as has been shown for dif here and by others [Wu and Anderson, 1998]. Only LPS stimulation induced nuclear translocation of RELISH.

In conclusion, we have demonstrated an LPS-induced processing event of *Drosophila* RELISH that coincides temporally with both the nuclear translocation of the active RHD domain and the production of antimicrobial peptides. In addition, this processing event appears to be cactus-independent since cactus is neither degraded nor necessary for induction of several antibacterial peptides by LPS. Furthermore and in contrast to dif, loss-of cactus expression does not induce nuclear translocation of RELISH. We propose a model for *Drosophila* innate immunity whereby LPS stimulation provides a signal to cells that results in the processing of RELISH protein and release of the C-terminal ankyrin repeats (Fig. 8). The N-terminal species of RELISH either as a homodimer or a heterodimer with other NF-κB molecules is free to translocate to the nucleus and mediate certain antimicrobial responses. Similarly, other immunostimulatory agents from bacteria could act in a cactus-dependent manner to induce cactus degradation and release of NF-κB molecules leading to nuclear translocation and the consequential induction of additional antimicrobial responses.

In the course of preparing this paper, a report appeared also demonstrating LPS induced cleavage of RELISH in S2 cells [Silverman et al., 2000]. Cleavage which was determined to be regulated at least in part through the phosphorylation by a novel I-κB Kinase comprised of IKKβ and IKKγ proteins. In addition, Stoven et al. [2000] showed a RELISH processing and nuclear translocation event in mbn-2 cells and whole flies. The conclusions drawn from these reports are largely consistent with those presented here. However, our data builds upon this by directly demonstrating that RELISH processing is not only tied to nuclear localization of RHD but also is a cactus-independent event. Taken together these results demonstrate that RELISH is key component of the LPS mediated antimicrobial response in *Drosophila* that is regulated by a mechanism distinct from other *Drosophila* NF-κB homologues.

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