NUCLEIC ACIDS, PROTEIN SYNTHESIS, AND MOLECULAR GENETICS:

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In Vivo Regulation of the IκB Homologue cactus during the Immune Response of Drosophila*

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The dorsoventral regulatory gene pathway (spätzle/Toll/cactus) controls the expression of several antimicrobial genes during the immune response of Drosophila. This regulatory cascade shows striking similarities with the cytokine-induced activation cascade of NF-κB during the inflammatory response in mammals. Here, we have studied the regulation of the IκB homologue Cactus in the fat body during the immune response. We observe that the cactus gene is up-regulated in response to immune challenge. Interestingly, the expression of the cactus gene is controlled by the spätzle/Toll/cactus gene pathway, indicating that the cactus gene is auto-regulated. We also show that two Cactus isoforms are expressed in the cytoplasm of fat body cells and that they are rapidly degraded and resynthesized after immune challenge. This degradation is also dependent on the Toll signaling pathway. Altogether, our results underline the striking similarities between the regulation of IκB and cactus during the immune response.

Transcription factors containing the Rel homology domain have been implicated in a number of developmental and physiological processes, including dorsoventral patterning and immune response in Drosophila, mammalian acute phase response, and lymphocyte differentiation (reviewed in Refs. 1–4).

In mammals, NF-κB is a generic name for a number of Rel proteins (p50, p52, RelA, and RelB), which associate as homo- or heterodimers (reviewed in Refs. 1 and 2). This transactivator plays a pivotal role in the regulation of immune and inflammatory response genes. NF-κB is retained in unstimulated cells in the cytoplasm by its inhibitor IκB and migrates into the nucleus after rapid degradation of IκB in response to activation by cytokines such as interleukin-1 and tumor necrosis factor α (reviewed in Refs. 1 and 2).

In Drosophila, the embryonic dorsoventral regulatory pathway comprises 12 known maternal effect genes (reviewed in Ref. 5). The end result of the activation of this pathway is the nuclear translocation of the Rel transcription factor Dorsal. Four components of this pathway, Toll (TL), Pelle (PLL), Cactus (CACT), and DORSAL (DL) are homologous to members of the interleukin-1 receptor/NF-κB pathway. The cytoplasmic domain of TL, a transmembrane receptor protein (6), is homologous to the cytoplasmic domain of the interleukin-1 receptor (7, 8). PLL (9) shares sequence homology with the interleukin-1 receptor associated kinase (10). DL (11) and CACT (12, 13) are homologous to NF-κB and IκB, respectively. Localized activation of the TL receptor in the ventral region of the embryo by its ligand, the spätzle (SPZ) protein, causes disruption of the DL-CACT complex and the subsequent nuclear translocation of DL and CACT (14, 15). Genetic and molecular analyses indicate that CACT, like IκB, is rapidly degraded in response to signaling (16–18).

The striking structural and functional similarities between NF-κB and DL signaling pathways have led to the proposal that they share a common ancestry (reviewed in Refs. 3 and 19).

Rel proteins have recently been shown to be involved in the immune response of Drosophila (reviewed in Ref. 4). In particular, it has been suggested that they control the induction of genes encoding antibacterial and antifungal peptides in the fat body and in blood cells. The upstream regions of these genes contain sequence motifs similar to NF-κB binding motifs of mammalian immune responsive genes (reviewed in Ref. 20). Experiments with transgenic flies have shown that these motifs are mandatory for immune inducibility of the insect antibacterial peptide genes (21, 22). Several Rel proteins were reported to be present in the fat body: DL (23), initially identified as the dorsoventral morphogen, DIF (for dorsal-related immunity factor; Ref. 24), and Relish, a NF-κB1 (p105)-like protein containing both Rel and ankyrin domains (25).

The precise roles of these Rel proteins in the control of these immune genes has not yet been clarified in vivo (26, 27). Recently, we have shown by genetic analysis that the intracellular components of the dorsoventral pathway (except for DL) and the extracellular TL ligand SPZ, collectively referred to as the TL pathway, control the expression of the antifungal peptide gene drosomycin in Drosophila adults (27). In flies carrying loss-of-function mutations in the pll, tub, Tl, and spz genes, the immune inducibility of the drosomycin gene is dramatically decreased. In contrast, in Tl gain-of-function mutants, in which the TL pathway is signal-independently activated, and in cactus-deficient mutants, the gene encoding drosomycin is constitutively expressed. Altogether, these data demonstrated that the TL/interleukin-1 receptor pathway is indeed an ancient regulatory cascade involved in the host defense of both mammals and insects (27).

The fat body of Drosophila provides a unique experimental system to dissect in vivo the TL/interleukin-1 receptor signaling pathway in the context of the immune response. In this study, we have focused our interest on the regulation of cactus, the last element of the genetically characterized cascade. We have first observed that the cactus gene is up-regulated in response to immune challenge and that the expression of cactus is controlled by the spätzle/toll gene regulatory cascade. We have also noted that two CACT isoforms are expressed in the cytoplasm of fat body cells and that they are rapidly degraded and resynthesized after immune challenge. This degradation is dependent on the TL signaling pathway.

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The results reported in this study were obtained with fat body extracted from either larvae or adults. The fat body, a functional analog of the mammalian liver, is the major site of antimicrobial peptide production in Drosophila. In larvae, it consists of a mass of large polyplid cells that can easily be dissected out. In contrast, adult fat body is a thin and loose tissue difficult to excise. Our analysis was performed with extracts of fat body cells and occasionally, when indicated, of adult abdominal carcass, which allows the extraction predominantly of fat body with minor contaminations from epidermal and muscle cells.

Expression of the cact Gene Is Induced in the Fat Body by Immune Challenge—In a previous study, we had observed that 3 h after a bacterial challenge, cact gene expression was markedly up-regulated in adults (27). We have now extended this study by analyzing the time course of cact gene expression both in excised larval fat body and in male adult carcass tissues. The Northern blot analysis, presented in Fig. 1 (A and B) shows a faint signal for cact transcripts in unchallenged fat body and adult carcass and a remarkably rapid and strong up-regulation following bacterial challenge. In both larvae and adults, peak values were observed after 2 or 3 h, after which the signals of cact transcripts leveled off. These kinetics of induction/up-regulation, frequently referred to as acute phase kinetics, were similar to those of the cecropin A gene in these experiments. In contrast, the drosomycin and the diptericin genes reached their highest level of expression only 6–16 h postchallenge (Fig. 1, A and B).

Two cact transcripts are observed during development; they are of approximately 2.2 kb (referred to as maternal/zygotic) and 2.6 kb (zygotic) and encode proteins of 71 and 69 kDa, respectively, which differ in their C-terminal part flanking the two oligonucleotides designed after the rp49 coding sequence; Ref. 39). The cecropin A1 probe cross-reacts with cecropin A2 transcripts (36).

**RESULTS**
Importantly, however, in the context of the present study, the cact gene is derived from Geisler et al. (13) and Kidd (12). The 1.2-kb transcript is unrelated to cact and may define the 5′ limit of the cact locus (13). The complete sequence of the cact genomic region was obtained from the Berkeley Drosophila Genome Project (accession number 49408). Coding regions are indicated as empty boxes. Sequences related to mammalian NF-κB response elements are indicated (●). The sequences of these motifs, given with their positions relative to the start site are as follows: motif 1, 5′-GGGATTTTT-3′; motif 2, 5′-GGGTTTAAAC-3′; motif 3, 5′-GGGTAAAC-3′; motif 4, 5′-GGGATTTTAC-3′. The sequences of these motifs, given with their positions relative to the start site are as follows: motif 1, 5′-GGGATTTTT-3′; motif 2, 5′-GGGTTTAAAC-3′; motif 3, 5′-GGGTAAAC-3′; motif 4, 5′-GGGATTTTAC-3′. The sequences of these motifs, given with their positions relative to the start site are as follows: motif 1, 5′-GGGATTTTT-3′; motif 2, 5′-GGGTTTAAAC-3′; motif 3, 5′-GGGTAAAC-3′; motif 4, 5′-GGGATTTTAC-3′. The sequences of these motifs, given with their positions relative to the start site are as follows: motif 1, 5′-GGGATTTTT-3′; motif 2, 5′-GGGTTTAAAC-3′; motif 3, 5′-GGGTAAAC-3′; motif 4, 5′-GGGATTTTAC-3′.

was strongly and persistently induced. As previously reported for the drosomycin gene (32), we also observed that Gram-positive bacteria were more potent inducers of the cact255 FZ reporter gene than Gram-negative bacteria (data not shown).

**cact Expression Is Autoregulated**—We have further analyzed the expression of the cact gene in Drosophila carrying mutations that affect the dorsoventral signaling pathway. We have first examined the expression of the cact255 FZ reporter gene in dominant gain-of-function Tl (TlD) and cact-deficient mutant larvae in which the TL pathway is signal-independently activated and the drosomycin gene is constitutively turned on (27). A first striking result, shown in Fig. 2, C and D, was that in both mutant contexts, the reporter gene was expressed in the absence of immune challenge in larvae. The level of β-galactosidase activity was higher than that induced by bacterial challenge in wild-type insects. Similar results were obtained in adult fat body (data not shown).

We have corroborated these results by Northern blot experiments. For this, total RNA was extracted from larval fat body and adult carcass of wild-type insects and of TlD and cact-
deficient Drosophila. The RNA was probed on Northern blots with cact and rp49 cDNAs. The cact mutation that we selected for these experiments was cact
- deficient (29), the strongest viable cact-deficient mutation (29). This mutation, which had been induced by ethyl methyl sulfonate treatment, does not alter the expression of the cact gene but rather seems to affect the biosynthesis of the CACT protein and leads to a weakly functional CACT (29, 34). The data presented in Fig. 5 confirm the results obtained with the cact
- deficient adults than in unchallenged controls. Altogether, our results demonstrate that the activation of the TL pathway is sufficient to trigger the expression of the cact gene. The constitutive expression of cact observed in cact-deficient mutants demonstrates that the cact gene is autoregulated.

We have next studied the inducibility of the cact gene in strains carrying strong loss-of-function mutations that are known to block the dorsoventral signaling pathway. In spz, Tl, tub
- , and pll
- mutant adults, the level of CACT inducibility after bacterial challenge was significantly lower than in wild-type adults; h and d, hours and days, respectively, after infection by B. bassiana.

Interestingly, in dorsoventral mutant larvae and adults. Using an anti-CACT monoclonal antibody, we performed a Western blot analysis of larval and adult fat body extracts and detected both the 69-kDa zygotic and the 71-kDa maternal/zygotic proteins, although the latter was less abundant and was not always detected (Fig. 6). We never observed the 72-kDa phosphorylated isofrom in the fat body. Note that we also observed that both CACT proteins are present in nearly all tissues in larval and adult fat body and in embryos are consistent with their putative function as a cytoplasmic inhibitor.

Earlier Western blot analyses of CACT protein expression had revealed three polypeptides, which are differentially expressed during development (Refs. 12 and 34; see also Fig. 6). In male extracts, two major proteins of 69 and 71 kDa cross-react with an anti-CACT monoclonal antibody (Refs. 12 and 34; Fig. 6). These proteins are also detected in female ovaries, where a third form of 72 kDa is present. The latter species is the major form of CACT in late stage oocytes and early embryos. Phosphatase treatment revealed that the 72-kDa protein is a phosphorylated form of the 71-kDa protein and that both are encoded by the 2.2-kb maternal/zygotic mRNA (12, 34, 40).

Using an anti-CACT monoclonal antibody, we performed a Western blot analysis of larval and adult fat body extracts and detected both the 69-kDa zygotic and the 71-kDa maternal/zygotic proteins, although the latter was less abundant and was not always detected (Fig. 6). We never observed the 72-kDa phosphorylated isofrom in the fat body. Note that we also observed that both CACT proteins are present in nearly all tissues in larval and adult fat body and in embryos are consistent with their putative function as a cytoplasmic inhibitor.

Bacterial Challenge Induces Degradation of CACT in Wild-Type Larvae and Adults—By Western blot analysis, we next studied the level of CACT proteins in the fat body during the immune response. Fat body from larvae and adults were collected at different time intervals after bacterial challenge. Fig. 7 (A and B) shows that in response to this challenge, both the 69- and 71-kDa forms were degraded. The signals corresponding to both protein bands decreased 30–90 min postchallenge.
We also observed that bacterial challenge apparently did not cause a rapid and transient depletion of the CACT pool, which is retained in the cytoplasm of the fat body by binding to the CACT protein. Our results indicate that the dissociation of this CACT-Rel complex is mediated by the TL signaling pathway. This autoregulatory loop allows for the rapid resynthesis of inhibitors, which can in turn shut down the response when the extracellular signal levels off (Fig. 8). In agreement with this hypothesis, several putative Rel binding sites are observed in the genomic region flanking the cactus gene. Interestingly, we have also observed that cactus expression is rapidly and markedly induced and, after a peak value at 3 h, gradually levels off, this profile of expression being evocative of that of mammalian acute phase response genes. Interestingly, we have also observed that cactus gene expression is controlled by the SPZ/Tl/CACT signaling pathway. Indeed, the activation of the TL signaling pathway in TlD gain-of-function and cactus-deficient mutants is sufficient for a strong induction of the cactus gene, whereas loss of function in any of the genes extending in the dorsoventral regulatory cascade from spz to pll results in a markedly impaired induction of the cactus gene by bacterial challenge. In contrast, the cactus gene remains fully inducible in imd mutants. In essence, the transcriptional profile of cactus in dorsoventral mutants parallels that earlier observed for the drosomycin gene (27). We hypothesize that both genes are induced via a Rel protein (possibly DIF or an as yet unidentified Rel protein, but not DL alone), which is retained in the cytoplasm of the fat body by binding to the CACT protein. Our results indicate that the dissociation of this CACT-Rel complex is mediated by the TL signaling pathway. This autoregulatory loop allows for the rapid resynthesis of inhibitors, which can in turn shut down the response when the extracellular signal levels off (Fig. 8). In agreement with this hypothesis, several putative Rel binding sites are observed in the genomic region flanking the cactus gene. Indeed, the observation that the expression of the cactus gene has a rapid and transient depletion of the CACT pool, which is retained in the cytoplasm of the fat body by binding to the CACT protein. Our results indicate that the dissociation of this CACT-Rel complex is mediated by the TL signaling pathway. This autoregulatory loop allows for the rapid resynthesis of inhibitors, which can in turn shut down the response when the extracellular signal levels off (Fig. 8). In agreement with this hypothesis, several putative Rel binding sites are observed in the genomic region flanking the cactus gene.}

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DISCUSSION

Transcriptional Regulation—In a previous study, we had shown that the genes encoding the components of the embryonic dorsoventral pathway are expressed at a low but detectable level in control adults. They are significantly up-regulated upon septic injury (27). The high transcriptional level of these genes in challenged insects obviously allows for amplification of antimicrobial peptide gene expression, by increasing the amount of SPZ/Tl/CACT components able to respond to the signal.

Here, we have analyzed in detail the kinetics of expression of the cactus gene during the immune response. We have found that cactus expression is rapidly and markedly induced and, after a peak value at 3 h, gradually levels off, this profile of expression being evocative of that of mammalian acute phase response genes. Interestingly, we have also observed that cactus gene expression is controlled by the SPZ/Tl/CACT signaling pathway. Indeed, the activation of the TL signaling pathway in TlD gain-of-function and cactus-deficient mutants is sufficient for a strong induction of the cactus gene, whereas loss of function in any of the genes extending in the dorsoventral regulatory cascade from spz to pll results in a markedly impaired induction of the cactus gene by bacterial challenge. In contrast, the cactus gene remains fully inducible in imd mutants. In essence, the transcriptional profile of cactus in dorsoventral mutants parallels that earlier observed for the drosomycin gene (27). We hypothesize that both genes are induced via a Rel protein (possibly DIF or an as yet unidentified Rel protein, but not DL alone), which is retained in the cytoplasm of the fat body by binding to the CACT protein. Our results indicate that the dissociation of this CACT-Rel complex is mediated by the TL signaling pathway. This autoregulatory loop allows for the rapid resynthesis of inhibitors, which can in turn shut down the response when the extracellular signal levels off (Fig. 8). In agreement with this hypothesis, several putative Rel binding sites are observed in the genomic region flanking the cactus gene. Indeed, the observation that the expression of the cactus enhancer trap insertion is inducible after microbrial challenge strongly suggests that this element is inserted in the vicinity of immune responsive regulatory sequences.

Our in vivo results establish a clear parallel with the regulation of IxBo in mammalian cell cultures (Fig. 8). Indeed, IxBo expression is up-regulated upon stimulation of cells with activators of NF-κB such as tumor necrosis factor α and phorbol 12-myristate 13-acetate or when cells are transfected with plasmids expressing various Rel proteins (41–45). The promoter of the IxBo gene contains several potential NF-κB binding sites, and the specific deletion of one of these sites, located 37 base pairs upstream of the TATA box, abolishes responses to phorbol 12-myristate 13-acetate and tumor necrosis factor in cell culture (43, 44).

Contrasting with IxBo and cactus regulation in the immune response, no transcriptional regulation of the cactus gene has been reported in the context of its involvement in dorsoventral axis formation. In the latter case, cactus mRNA and proteins are synthesized during oogenesis and accumulate in the eggs (12, 13, 34). One should keep in mind that in contrast to the antimicrobial response, the formation of the dorsoventral gradient is a short process (a few hours) and is developmentally pro-
The Western blot analysis of the fluctuations of CACT protein in the fat body following bacterial challenge points to several successive phases. In control insects, both CACT isoforms are expressed at a low level, the 69-kDa protein being predominant in the fat body.

The observations that bacterial challenge failed to induce the depletion of CACT in the 71-kDa form) is detected. The observation that bacterial challenge; Refs. 23 and 46) are in excess of that of the inhibitor IκBα, which can reportedly enter the nucleus and inhibit the DNA binding of mammalian Rel proteins (47, 48). The latter mechanism has not been thoroughly analyzed in Drosophila, and no CACT nuclear localization has been reported in the early embryonic syncytium (17, 34) and in the fat body cells (this study).

In mammals, it has been proposed that other IκB members with distinct regulatory properties (e.g. IκBβ) could be involved in the persistent activation of Rel proteins (49). We cannot exclude the possibility that either other as yet unidentified CACT-like members in Drosophila or the NF-κB1 (p105)-like Relish protein (25) containing both Rel and ankyrin domains could also inhibit Rel proteins. But the observation that the Rel proteins are nuclear in cact-deficient mutants suggests that no other inhibitor(s) can fully rescue the absence of CACT.

Conclusions—In Drosophila, as in other organisms, signal transduction pathways are involved in various developmental and physiological processes. These cascades exhibit subtle differences to account for their respective functions in these tissues. The TL signaling pathway, which is involved in embryonic dorsoventral patterning, in the antimicrobial response, and probably in several other processes (reviewed in Ref. 3) is a good example. It is interesting in this context to note that in contrast to embryonic development, the regulation of CACT in the fat body involves an autoregulatory loop.

Finally, the data in this paper reveal striking functional similarities between transcriptional and post-translational regulation of IκBα and CACT (Fig. 8). This strengthens the idea that the signaling pathways activating Rel proteins during the host defense have been conserved between insects and mammals. The powerful genetic system of Drosophila provides an excellent model to further dissect the control mechanisms of IκB/Rel activation.

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REFERENCES