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/leucine-rich repeat protein (LRP) gene) 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/leucine-rich repeat protein (LRP) 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 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 (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 plf, tub, Tf, and spz genes, the immune inducibility of the drosomycin gene is dramatically decreased. In contrast, in Tf gain-of-function mutants, in which the TL pathway is signal-independent activated, and in cact-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 cact, the last element of the genetically characterized cascade. We have first observed that the cact gene is up-regulated in response to immune challenge and that the expression of cact is controlled by the spz/Tlicact 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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**EXPERIMENTAL PROCEDURES**

**Drosophila Stocks**—The cact<sup>255</sup> strain contains an FZ enhancer trap (28) in the first intron of the cact gene. The cact<sup>255</sup> FZ line exhibits an embryonic pattern of lacZ expression similar to that of the resident cact gene as detected by in situ hybridization of its transcripts (12). This insertion causes a strong CACT phenotype (13, 29). <sup>T</sup><sub>10b</sub> and <sup>T</sup><sub>79b</sub> are two dominant gain-of-function ventralizing alleles of Toll (<sup>T</sup><sub>l</sub>) caused by a single amino acid change (30). Other dorsoventral mutant stocks used in this study have been described elsewhere (26, 31). All experiments were performed at 25 °C except when otherwise stated.

**Infection Experiments**—Bacterial challenges were performed by prickling third instar larvae or adults with a needle dipped into a concentrated culture pellet of *Escherichia coli* and *Micrococcus luteus* (OD of the pellet ~ 100). Natural infection with entomopathogenic fungi was performed by shaking anesthetized flies for a few minutes in a Petri dish containing a sporulating culture of *Beauveria bassiana* (strain 80.2). Flies covered with spores were then placed onto fresh *Drosophila* medium and incubated at 29 °C. Natural infection with entomopathogenic fungi induces a strong and sustained expression of the antifungal peptide gene *drosomycin*, through the selective activation of the TL signaling pathway (32).

**β-Galactosidase and Immunolocalization Stainings**—The β-galactosidase activity measurement and staining method were as described in Ref. 33. Immunolocalization experiments were performed as in Ref. 26. A monoclonal anti-CACT mouse antibody (2C2–50; Ref. 34) was applied on the fat bodies at a 1:100 dilution. The second antibody was an alkaline phosphatase-linked sheep anti-mouse-IgG (Boehringer Mannheim) diluted 1:500.

**RNA Preparation and Analysis**—Croses were performed at 25 °C, and third instar larvae or 2–4-day-old adult flies were collected. Total RNA was extracted from dissected larval or adult fat body with the RNA Trizol (Life Technologies, Inc.) method. Total RNA extraction and Northern blotting experiments were performed as in Ref. 35. The following probes were used: cecropin A1 cDNA (36), diptericin cDNA (37), drosomycin cDNA (38), a CACT cDNA (a polymerase chain reaction product of approximately 1.5 kb<sup>1</sup> corresponding to the N-terminal part of cact), and rp49 cDNA (a polymerase chain reaction fragment of approximately 400 base pairs generated between two oligonucleotides designed after the rp49 coding sequence; Ref. 39). The cecropin A1 probe cross-reacts with cecropin A2 transcripts (36).

**Western Blot Analysis**—The monoclonal anti-DL antibody (7A4–25, 34) used in this study is directed against the C-terminal domain of the DL protein. The monoclonal anti-CACT antibody 2C2–50 was described by Whalen and Steward (34). A monoclonal anti-β-tubulin antibody (Boehringer Mannheim) was used as a loading control. Larval or adult fat bodies from 30–40 insects were collected and frozen at ~80 °C. Fat bodies were lysed in 2× Laemmli solution. 15 μg of fat body extract were loaded on a 7.5% SDS-polyacrylamide gel. Following SDS-polyacrylamide gel electrophoresis, proteins were blotted to Hybond ECL nitrocellulose membranes (Amersham Life Science). The blots were developed using the ECL system (Amersham) and x-ray film to detect the signal. Cylcholeoximide treatment was performed by injecting ~20 μl of a mixture of cylcholeoxime (10 μg/ml) and bacterial suspension into the thorax of *Drosophila* adults using a Nanoject apparatus (Drummond<sup>TM</sup>.

**RESULTS**

The results reported in this study were obtained with fat body extracts 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 polyloid 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 PEST sequence (12, 13). The above Northern blot analyses were performed with total RNA, a method that does not accurately discriminate between these two transcripts. We have therefore repeated the analysis with Northern blots prepared with poly(A) RNA from total adults; as shown in Fig. 1C, both transcripts were detectable in unchallenged tissues and were clearly up-regulated after bacterial challenge, the 2.2-kb transcript being predominant.

We have further investigated cact expression by using an enhancer trap line, cact<sup>255</sup> FZ, in which an FZ element is inserted in the first intron of the cact gene (12, 13). The FZ element is a P transposon containing the minimum *fushi tarazu* (ftz) promoter fused to lacZ and behaves like a PZ enhancer trap except that β-galactosidase expression is predominantly cytoplasmic (28). The cact<sup>255</sup> FZ insertion causes a strong cact phenotype, and homozygous cact<sup>255</sup> mutants die.

<sup>1</sup>The abbreviation used is: kb, kilobase pair(s).
Importantly, however, in the context of the present study, gene expression during natural infections by the entomopathogenic fungus B. bassiana. For this, we have covered cact255 FZ adults with B. bassiana spores and measured lacZ expression over a 6-day period. Fig. 4 shows that under these conditions, which mimic a natural infection, the cact255 FZ reporter gene 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 Ti (TITd) 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 TITd 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*<sup>255</sup>, 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*<sup>255</sup> *FZ* reporter gene approach; the level of *cact* transcripts was indeed significantly higher in *T<sup>D</sup>* and *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<sup>−</sup>, T<sup>−</sup>, tub<sup>−</sup>, and *pll<sup>−</sup>* mutant adults, the level of CACT inducibility after bacterial challenge was significantly lower than in wild-type adults; *h<sup>−</sup> and d<sup>−</sup>* adults, hours and days, respectively, after infection by *B. bassiana*.

### Regulation of *cactus* during *Drosophila* Immune Response

We have next studied the inducibility of the *cact* gene in wild-type and dorsoventral mutant larvae and adults. By Western blot analysis, we next observed that both CACT proteins are present in nearly all tissues in larvae and adults (data not shown). These data indicating that *CACT* proteins have similar subcellular localizations in the 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; data not shown). These data indicating that CACT proteins have similar subcellular localizations in the fat body and in embryos are consistent with their putative function as a cytoplasmic inhibitor.

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 protein, which 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). 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).

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### 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. The results indicate that both protein bands decreased 30–90 min postchallenge.
We also observed that bacterial challenge apparently did not yield an anti-CACT signal. A higher amount of the 71-kDa form was more abundant (Fig. 7, panels E and F) than in wild-type unchallenged controls, except that the latter never totally disappeared. The kinetics of degradation were essentially similar to those observed for I-kB in cell culture (41).

Injection of cycloheximide prior to bacterial challenge in adults prevented the reappearance of both migrating species, indicating that protein synthesis was required (Fig. 7D). Altogether, these results indicate that immune challenge induces in vivo a rapid and transient depletion of the CACT pool, which is regenerated by de novo synthesis.

TI Controls the Immune Induced Degradation of CACT—We have also examined the immune induced degradation of CACT in larvae and adults carrying mutations that alter the dorsoventral signaling pathway. No immune induced degradation of CACT was observed in fat body extracts derived from TI-deficient mutants (Fig. 7, E and F), indicating that the immune induced degradation of CACT requires the TL signaling cascade. It should be noted that, in contrast to adults, only the 69-kDa zygotic CACT form was detected in TI larvae (Fig. 7E).

We next analyzed the level of CACT protein in fat body extracts derived from TID mutants. As stated above, in this background, both DL and DIF are nuclear (24, 26), and the drosomycin gene is constitutively turned on (27). Surprisingly, in TID flies, the level of CACT protein was similar to or even higher than in wild-type unchallenged controls, except that the amount of 71-kDa protein was more abundant (Fig. 7, H and I). We also observed that bacterial challenge apparently did not induce CACT degradation in TID mutants. However, cycloheximide treatment of TID adults resulted in a loss of CACT (Fig. 7, J). Interestingly, the same level of CACT proteins (with a higher amount of the 71-kDa form) was detected in fat body extracts from adults that had been infected with entomopathogenic fungi (Fig. 7, C, Fg). The situation observed in TID may therefore represent the in vivo level of CACT in persistent infections.

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 cact gene during the immune response. We have found that cact 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 cact gene expression is controlled by the SPZ/TL/CACT signaling pathway. Indeed, the activation of the TL signaling pathway in TID gain-of-function and cact-deficient mutants is sufficient for a strong induction of the cact 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 cact gene by bacterial challenge. In contrast, the cact gene remains fully inducible in imd mutants. In essence, the transcriptional profile of cact 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 cact gene (255 FZ insertion site. Indeed, the observation that the expression of the cactFZ enhancer trap insertion is inducible after microbial 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 IκBα in mammalian cell cultures (Fig. 8). Indeed, IκBα 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 IκBα 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 IκBα and cact regulation in the immune response, no transcriptional regulation of the cact gene has been reported in the context of its involvement in dorsoventral axis formation. In the latter case, cact 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-
grammed. Consequently it may not require a renewed transcription of the dorsoventral genes and the synthesis of the corresponding protein products.

Post-translational Regulation—We have detected two CACT isoforms of 69 and 71 kDa in the fat body but did not observe the 72-kDa phosphorylated CACT species, which is the predominant form in late ovaries and early embryos. We have no idea whether the two CACT isoforms have distinct regulatory properties in the control of antimicrobial peptide gene expression. However, in agreement with previous studies (12, 16), our data indicate that the 69-kDa protein, encoded by the 2.6-kb maternal/zygotic transcript, is more stable than the maternal form; although the maternal 2.2-kb mRNA is more abundant than the 2.6-kb zygotic transcript, the 69-kDa protein is predominant in the fat body.

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 response to immune challenge, a rapid depletion of both CACT isoforms is observed with the maternal/zygotic 71-kDa species disappearing completely. This CACT degradation is mediated by the TL signaling pathway as demonstrated by the fact that it does not occur in Tl-deficient mutants. This short depletion phase (30–90 min) is rapidly followed by the regeneration of both isoforms by de novo synthesis, as illustrated by our cycloheximide studies. During this phase, the CACT levels reach an equilibrium between signal-induced degradation and de novo synthesis of CACT following intense expression of its gene. A similar situation is observed in Tp mutants, where the TL pathway is constitutively activated and where a high level of CACT protein (particularly of the 71-kDa form) is detected. The observation that bacterial challenge failed to induce the depletion of CACT in Tp mutants also suggests that a state of equilibrium has been reached under constitutive signaling. We may anticipate that in wild-type challenged animals, at a later stage, the decrease of signaling is correlated with a return to the normal situation.

The findings that Tp mutants or persistently infected adults express high titers of CACT are at first sight paradoxical, since in these backgrounds the Rel protein DIF and DL are predominantly nuclear (24, 26) and the drosomycin gene is constitutively turned on (27). Several explanations can account for the activation of Rel proteins in the presence of a high level of inhibitor. One possibility is that the levels of Rel proteins (the dl and dif genes are themselves up-regulated upon bacterial challenge; Refs. 23 and 46) are in excess of that of the inhibitor CACT. Alternatively, we propose that the nuclear translocation of the Rel proteins is not strictly correlated to the level of CACT proteins but rather to the intensity of CACT degradation. This implies that once dissociated from the Rel-CACT complexes, the Rel proteins cannot be inhibited by free CACT (e.g. because of structural modifications). Such a model would ensure a strict correlation between the level of signaling and the level of Rel nuclear translocation. However, it excludes the possibility of an active inhibitory mechanism by CACT of the cognate Rel proteins, in contrast to IxBα, 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 IxB members with distinct regulatory properties (e.g. IxBβ) 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-kB1 (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 IxBα 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 IxB/Rel activation.

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