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SIGNALS FROM THE IL-1 RECEPTOR HOMOLOG, TOLL, CAN ACTIVATE AN IMMUNE RESPONSE IN A *DROSOPHILA* HEMOCYTE CELL LINE

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The *Toll* gene encodes an interleukin 1 receptor-like protein that mediates dorsoventral polarity in the *Drosophila* embryo. The possible involvement of Toll or Toll-like proteins also in the *Drosophila* immune response was investigated by overexpressing $Toll^{10B}$, a constitutively active mutant protein, in the *Drosophila* blood cell line mbn-2. Induction of the *Cecropin A1* (*CecA1*) gene, coding for a bactericidal peptide, was used as an indicator for the immune response. $Toll^{10B}$ was found to increase *CecA1* transcription, as detected with a cotransfected *CecA1-lacZ* reporter gene construct. This effect depends on the presence of a κB-like site in the *CecA1* promoter. The endogenous *Toll* gene is expressed in mbn-2 cells, indicating that this gene may normally play a role in *Drosophila* blood cells.

The molecular basis for the recognition of non-self in insects is unknown. Clonally selected recognition molecules like immunoglobulins and T cell receptors are lacking, and there is no immunological memory. Still, insects mount an efficient immune response when they are infected, and a series of antibacterial proteins and peptides is induced, including the cecropins, amphipathic peptides that attack bacterial cell membranes (1-4). This immune response may be related to innate immune reactions in vertebrates.

The transcription factor NF-κB and other members of the Rel family play an important role in mammalian immunity (5,6). Similarly, insects depend on transcription factors of the Rel family for the activation of their immune response. From *Drosophila*, two Rel proteins have been described so far. One of them,

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Abbreviations: IL-1, interleukin-1; IL-1R, interleukin-1 receptor.

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dorsal-related immunity factor (Dif), is involved in the transcriptional activation of the cecropins (7). The other *Drosophila* Rel protein, dorsal, is best known for its involvement in the determination of dorsoventral polarity in the early embryo (8,9), but this protein has also been implicated in the induction of another antibacterial protein, diptericin (10).

There are several interesting parallels between the signaling pathways used to establish dorsoventral polarity in the *Drosophila* embryo, and those that activate parts of the mammalian immune system (9). NF-κB and dorsal are both Rel proteins that normally rest in an inactive form in the cytoplasm, bound to ankyrin-related inhibitory proteins, IκB and cactus, respectively. Upon activation, the inhibitory proteins are degraded and the active transcription factors become relocated to the nucleus. Furthermore, the membrane receptor Toll, that mediates signals to activate dorsal in the *Drosophila* embryo, is related both to the interleukin 1 receptor (IL-1R), that activates NF-κB (11), and to the macrophage differentiation marker MyD88 (12,13).

It is possible that the signaling pathways of insect immune responses are related to those involved in the activation of NF-κB and dorsal. In $Toll^{10B}$ mutant flies, which carry a constitutively activated Toll protein, both Dif and dorsal have been found to be localized in the nucleus rather than the cytoplasm, even in the unstimulated animal (7,10,14). Here, we have studied the effect of the Toll protein on the induction of the cecropin gene promoter, using the immunoresponsive *Drosophila* blood cell line mbn-2 (15).

MATERIALS AND METHODS

Cell culture–The mbn-2 cell line, described in (16), was a kind gift from E. Gateff. Cells were grown in Schneider's medium (NordCell, Stockholm, Sweden) supplemented with 5% heat-inactivated fetal calf serum, 1x Glutamax I (GIBCO), 50 U/ml penicillin, 50 μg/ml streptomycin and 50 μg/ml gentamicin.

Plasmids–F9-Toll and F9-$Toll^{10B}$ were a gift from K. Kubota and N. J. Gay. They contain the wild type Toll and the dominant $Toll^{10B}$ cDNAs , respectively, under the control of the inducible heat-shock promoter (17,18). The *CecA1-lacZ* fusion constructs have been described elsewhere (19, Petersen, Björklund, Ip, and Engström, submitted). Briefly, pA10 contains 822 bp upstream sequence from the *Drosophila CecA1* gene. In pA15 the -105/-69 fragment was removed, deleting the κB-like motif. The p(attκB)$_3$ construct contains a trimer of the attacin κB-like motif from *H. cecropia*, fused to a minimal 68 bp *CecA1* promoter. The p(attκBmut)$_3$ contains a trimer of a mutated κB-like motif, inserted at the same position. The transfection efficiency control pPacCAT, with the chloramphenicol acetyl transferase gene fused to a constitutive actin promoter, was constructed by K. Furukubo-Tokunaga and is described in (19). All plasmids used for transfections were purified by cesium chloride centrifugation.

Transfections–Cells were transfected by the calcium-phosphate precipitation method (20), typically using 10 μg of the respective Toll construct, 1 μg of the *lacZ* reporter gene construct, 2 μg of the transfection efficiency control pPacCAT and a carrier plasmid DNA to a final amount of 20 μg. At about 48 h after transfection, the expression of Toll protein was induced by heat-shock (30 min at 37°C) and the cells were allowed to recover for 90 min at 25°C. The cells were then incubated

with purified LPS (10 μg/ml) for 4 h. After lysis, β-galactosidase and CAT activity in the extracts was analysed as described (19). β-galactosidase activity was normalized to CAT activity to correct for variations in transfection efficiency.

RNA extraction and analysis–RNA was extracted from the cells as described in (21). 10 μg were loaded on a formaldehyde gel, blotted on a nitrocellulose filter (Costar), and probed with a Toll cDNA labelled by random priming. Hybridization was performed in 2x SSC at 65°C.

RESULTS AND DISCUSSION

Plasmids that express either wild-type Toll or mutant Toll^{10B} protein from a heat shock promoter were constructed by Kubota *et al.* (18) to study the interaction between Toll and dorsal. We used the same constructs to investigate their effect on the activity of the *CecA1* promoter.

Fig. 1 shows that the constitutively active Toll^{10B} mutant protein causes increased β-galactosidase expression from pA10, a *CecA1-lacZ* reporter gene construct, when coexpressed in mbn-2 cells. As previously described (15,19), LPS also stimulates *CecA1* transcription. However, in the transfection experiments there is a considerable background of expression even in the controls, since the transfection protocol itself induces the expression of *Cec* genes (19). The relative increase caused by Toll^{10B} varies from experiment to experiment but is independent of LPS concentration, even at 10 μg/ml, the maximally stimulating level (15). Overexpression of the Toll wild-type construct gives little or no effect, whereas Toll^{10B} gives maximal stimulation even in the absence of heat shock (data not shown), presumably due to leaky expression from the heat shock promoter (18), or when the amount of expression construct used for transfection is reduced from 10 to 2 μg (see legend to Fig. 2).

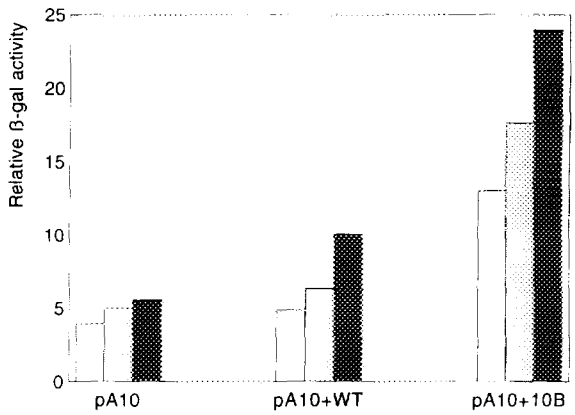

Fig. 1. Effect of Toll and Toll^{10B} overexpression on the *CecA1* promoter. β-galactosidase activities (adjusted for transfection efficiency) in extracts of cells transfected with the pA10 reporter construct alone, or pA10 together with 10 μg of the wild-type or Toll^{10B} expression constructs. Cells were incubated without LPS (white bars), with 10 ng/ml LPS (grey bars), or with 10 μg/ml LPS (black bars).

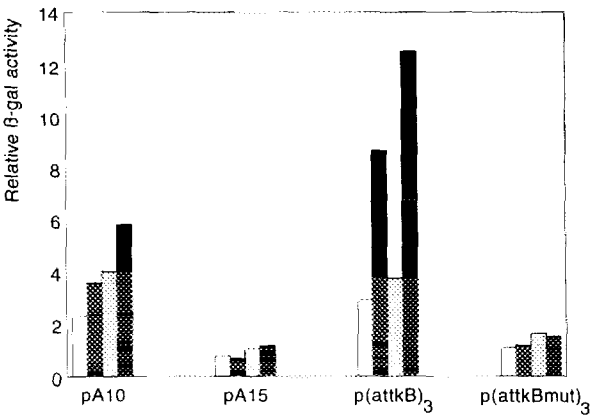

Fig. 2.Requirement of a κB-like promoter element for stimulation by Toll^{10B}. β-galactosidase activities (adjusted for transfection efficiency) in extracts of cells cotransfected with different reporter constructs and either the wild-type Toll expression construct without (white bars) or with (dark grey bars) induction by 10 μg/ml LPS, or the Toll^{10B} expression construct without (light grey bars) or with (black bars) induction by 10 μg/ml LPS. Similar results were obtained when 2 or 10 μg of the expression constructs was used, and the results from these experiments have been averaged.

The stimulatory activity of LPS on the *CecA1* promoter depends on the presence of a *cis*-acting κB-like element that serves as a binding site for Dif (7,19). The effect of Toll^{10B} appears to require the same element. In the plasmid construct pA15, where the κB-like element is deleted, the stimulatory effect of Toll^{10B} is largely abolished (Fig. 2). Similarly, the p(attκB)$_3$ construct, where a κB trimer is linked to a minimal promoter, is stimulated both by LPS and Toll^{10B}, whereas the control p(attκBmut)$_3$, with a mutated κB trimer, remains inactive.

These experiments demonstrate that the *CecA1* promoter has the capacity to respond to signals from the Toll protein. A similar signaling pathway may operate during the normal immune response in *Drosophila*, involving either Toll or related proteins. That Toll itself may play a role is indicated by the fact that the endogenous *Toll* gene is actively transcribed in the *Drosophila* blood cell line mbn-2, as shown in Fig. 3. This expression is not further enhanced by LPS.

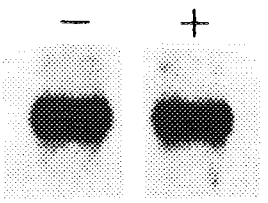

Fig. 3.*Toll* expression in the hemocyte cell line mbn-2. Northern blot analysis of Toll expression, with RNA extracted from mbn-2 cells incubated without (-) or with (+) 10 μg/ml LPS.

The effect of LPS on *Cec* gene expression is probably not mediated by the Toll protein since $Toll^{10B}$ stimulates *CecA1-lacZ* transcription even after maximal stimulation with LPS, and conversely, LPS is stimulatory in a situation where further $Toll^{10B}$ expression by heat shock has no effect. Rather, LPS and Toll may act via independent pathways that converge on the κB-like element in the *CecA1* promoter. The fact that the antibacterial genes were not induced in $Toll^{10B}$ mutant flies (10) indicates that additional signals may be required, or that a negative feedback control is activated under conditions of chronic stimulation. This question can not easily be addressed in the mbn-2 cell system, since the *Cec* genes are already partly induced in the transfected cells.

Our experiments show that a Toll-like protein, possibly Toll itself, may modulate antibacterial responses in insects. It can not be excluded that the *Toll*-related gene, *18w*, is also involved, and mutants in both genes increase the frequency of melanotic "tumors", believed to represent a pathological activation of the immune system (22,23). A Toll/IL-1R-like protein was recently shown to mediate viral resistance in tobacco (24), and the involvement of these proteins in immune reactions may be of very old date.

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