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## REVIEW

# NF- $\kappa$ B signaling pathways in mammalian and insect innate immunity

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## Innate immunity

Innate immunity is the first line of defense against infectious microorganisms. The innate immune system relies on germ line-encoded pattern recognition receptors (PRRs) to recognize pathogen-derived substances (Janeway 1989). Activation of the innate immune system through these receptors leads to the expression of a vast array of antimicrobial effector molecules that attack microorganisms at many different levels. The innate immune system appeared early in evolution, and the basic mechanisms of pathogen recognition and activation of the response are conserved throughout much of the animal kingdom (Hoffmann et al. 1999).

In contrast to innate immunity, the adaptive immune system generates antigen-specific receptors, antibodies, and T-cell receptors by somatic cell DNA rearrangement. These receptors, found only in higher eukaryotes, recognize specific pathogen-encoded proteins. Mammals have a complex immune response, which relies on communication between the innate and adaptive arms of the immune system. The innate immune response generates a costimulatory signal, which is required in combination with antigen-specific recognition to activate T-cells and the adaptive immune system. Antigen-specific recognition in the absence of costimulation can lead to anergy rather than activation (Janeway 1989). Thus, the activation of an antigen-specific response is coupled to infection through the innate immune system.

Insects have a very potent innate immune response that effectively combats a broad spectrum of pathogens. For example, *Drosophila* can withstand, and clear, bacterial burdens that, relative to their size, would be lethal to mammals (Hoffmann and Reichhart 1997). Induction of innate immunity in both mammals and insects leads to the activation of similar effector mechanisms, such as stimulation of cell-based phagocytic activity and expression of antimicrobial peptides (Hoffmann et al. 1999). For example, *Drosophila* produce a wide range of potent antimicrobial peptides in response to infection by fungi or bacteria (Hoffmann and Reichhart 1997). Induction of

the antimicrobial peptides is regulated at the level of transcription, and they are expressed primarily in the fat body, the insect liver analog.

Recent studies have revealed striking similarities in the signaling pathways used by humans and flies to activate their innate immune responses. In both cases, infection leads to the activation of Toll-like receptors (TLRs), which in turn initiate intracellular signaling cascades that culminate in the activation of NF- $\kappa$ B/Rel family transcription factors. In this review, we discuss recent advances in understanding the signaling pathways in mammalian and *Drosophila* innate immunity, with emphasis on the mechanisms by which NF- $\kappa$ B/Rel family proteins are activated.

## Innate immune signaling pathways

### *Drosophila*

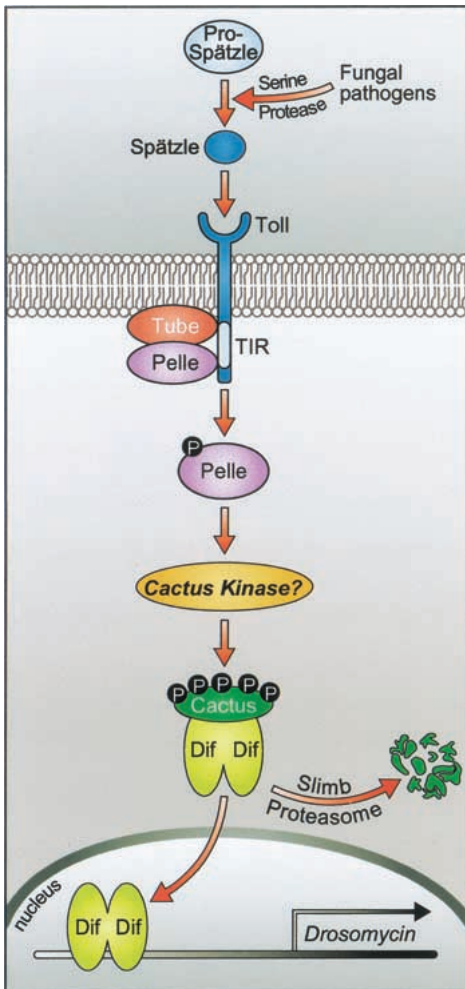
*Drosophila* has two independent immune signaling pathways, both of which lead to the activation of NF- $\kappa$ B transcription factors. One pathway responds primarily to fungal and gram positive bacterial infection (Fig. 1), while the other responds to lipopolysaccharide (LPS) treatment or infection by gram-negative bacteria (Fig. 2) (Lemaitre et al. 1996, 1997). The antifungal pathway requires components of the Toll signaling pathway, most of which are also required during dorsoventral patterning of the embryo (Belvin and Anderson 1996). Induction of the Toll/antifungal pathway leads to the activation of two *Drosophila* NF- $\kappa$ B homologs, called Dorsal and Dif, resulting in the production of antifungal peptides such as Drosomycin. Dorsal is also required in early embryogenesis for the Toll-dependent patterning of the dorsoventral axis. Dif is required for antifungal immunity in the adult fly, whereas in larvae either Dif or Dorsal is sufficient for the immune response (Manfruelli et al. 1999; Meng et al. 1999; Rutschmann et al. 2000a).

By contrast, the LPS-inducible antibacterial pathway requires the third *Drosophila* NF- $\kappa$ B homolog, Relish, which activates the expression of genes encoding antibacterial peptides such as Diptericin (Hedengren et al. 1999; Rutschmann et al. 2000b). Relish is synthesized as an NF- $\kappa$ B precursor protein that is cleaved and activated in response to bacterial infection or LPS treatment (Stöven et al. 2000). Interestingly, some antimicrobial

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**Figure 1.** The *Drosophila* Toll/antifungal signaling pathway. This model highlights current understanding of the Toll signaling pathway as it functions during the immune response. The pattern recognition receptors that recognize fungal pathogens are unknown, but they are believed to activate a serine protease cascade, culminating in the cleavage of the Toll ligand Spätzle. Ligand binding to Toll leads to the recruitment of two proteins, the adaptor Tube and the kinase Pelle. Recruitment of Pelle is thought to cause its activation and disassociation from Toll. Activated Pelle may then activate, directly or indirectly, a Cactus kinase that is responsible for signaling the proteasome-mediated degradation of Cactus. Currently, the biochemical steps between Pelle and Cactus degradation remain undetermined, and the Cactus kinase has not yet been identified.

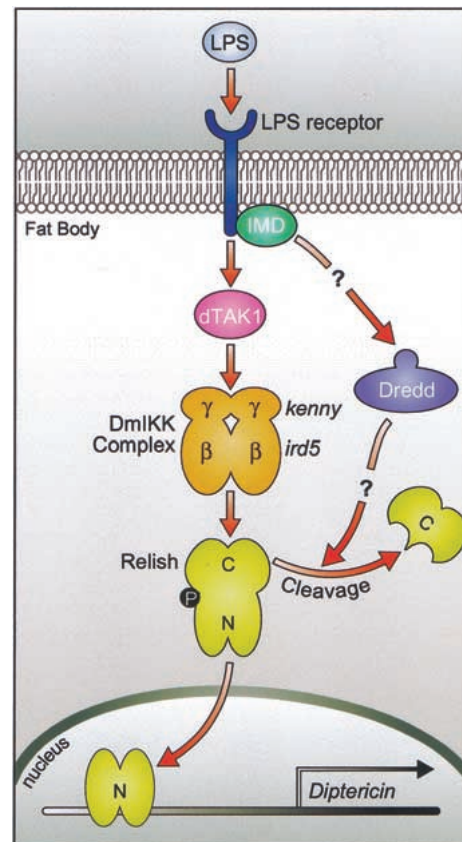
peptides, such as Cecropin, can be activated by either pathway and are thought to have both antibacterial and antifungal activities (Lemaitre et al. 1996; Ekengren and Hultmark 1999). In any case, one mechanism used to direct the transcription apparatus to distinct sets of antimicrobial genes in response to different pathogens is the activation of different members of the NF- $\kappa$ B family.

In *Drosophila*, infection also leads to the activation of the JNK (Sluss et al. 1996) and JAK/STAT signaling pathways (Lagueux et al. 2000). The function of the JNK path-

way in immunity has not been established whereas the JAK/SAT pathway is necessary for the induction of a number of complement-like proteins that have recently been shown to function by opsonizing gram-negative bacteria and promoting their phagocytosis (Lagueux et al. 2000; Levashina et al. 2001). Very little is known about the response of the insect immune system to other classes of pathogens, for example, virus, spirochetes or plasmodia.

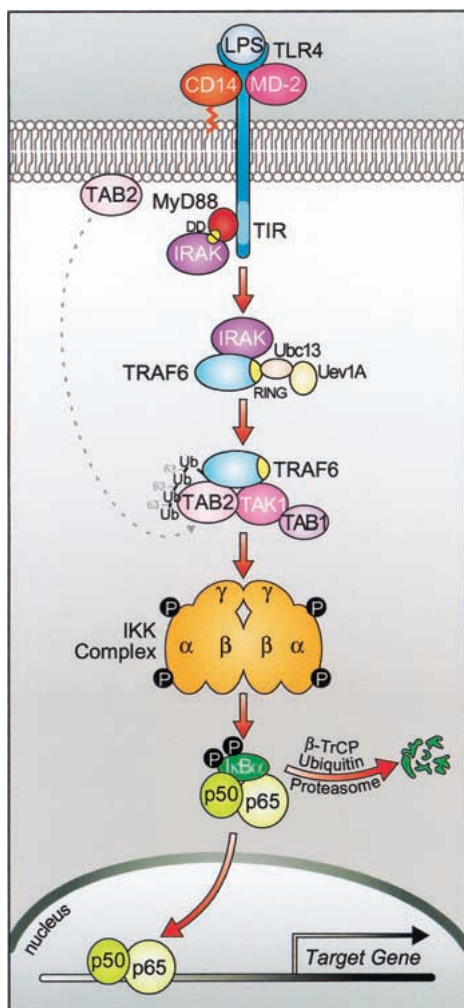
### Mammals

The mammalian innate immune system responds to a plethora of microbial-derived substances including microbial cell wall components such as LPS, peptidylglycans, and lipoproteins (Krutzik et al. 2001). The innate immune system can also be activated by bacterial DNA (Krieg 1996) or double-stranded RNA, the latter of which



**Figure 2.** The *Drosophila* antibacterial signaling pathway. In this model the signaling pathway is activated by LPS through unidentified receptor(s) and leads to Relish cleavage. Downstream of the receptors, this signaling pathway bifurcates. One part leads to activation of the *Drosophila* IKK complex, which then phosphorylates Relish. The other part functions through the caspase Dredd and leads to the cleavage of phosphorylated Relish. At present it is not known whether Dredd acts directly or indirectly to cleave Relish. The IMD protein may function in one or both of these pathways. (N) Amino-terminal domain; (C) carboxy-terminal domain.

is common to many viruses (Mogensen and Paludan 2001). The TLRs play a central role in the recognition of many of these immunostimulatory molecules and are probably responsible for the recognition of most types of pathogens. In response to these signals, the TLRs activate signaling pathways that culminate in the expression of antimicrobial molecules (proteins, peptides, and reactive oxygen and nitrogen intermediates), cytokines, and costimulatory molecules (Fig. 3) (Medzhitov et al. 1997;



**Figure 3.** The LPS signaling pathway in mammals. In this model LPS is recognized by a complex of three proteins, CD14, MD-2, and TLR4. TLR4 activates the intracellular signaling cascade by recruiting MyD88 and IRAK to the membrane. IRAK associates with the receptor complex transiently; once released IRAK can associate with and activate TRAF6. The TRAF6 RING finger, in combination with Ubc13 and Uev1A, mediates the K63-extended polyubiquitination of TRAF6 itself. The TAK1/TAB1/TAB2 complex is activated by its association with ubiquitinated TRAF6. Interestingly, the TAK1-associated protein TAB2 translocates from the membrane fraction to the cytoplasmic fraction upon treatment with IL-1. Once activated, the TAK1 complex phosphorylates and activates the IKK complex. The activated IKK complex then phosphorylates I $\kappa$ B $\alpha$ , leading to its ubiquitination and degradation by the proteasome.

Thoma-Uszynski et al. 2001; Zhang and Ghosh 2001). Thus, activation of the innate immune system immediately slows infection and activates other aspects of the immune system, primarily T-cells. The TLRs activate a number of signaling pathways including the JNK/AP-1 pathway, proapoptotic caspase cascades, and NF- $\kappa$ B inducing pathways (Medzhitov et al. 1997; Muzio et al. 1998; Aliprantis et al. 1999, 2000). These pathways are responsible for activating the appropriate effector mechanisms and signaling molecules.

The innate immune system plays a critical role in regulating the decision between the two types of mammalian immune responses, referred to as Type 1 and Type 2 responses. The Type 1 response functions to combat small intracellular pathogens such as bacteria whereas the Type 2 response combats larger extracellular pathogens such as helminths. The particular array of signaling molecules expressed by antigen presenting cells (APCs) determines whether a Type 1 or Type 2 response is activated. The expression of these signaling molecules, cytokines and costimulatory molecules, is controlled by the types of pathogens sensed by APCs through PRR, such as the TLRs. Thus, in addition to its role in activating the adaptive immune system, the innate immune response plays a central role in coordinating the particular type of adaptive immune response so that it will be most effective in combating the pathogens presented (Pulendran et al. 2001).

These two types of responses in mammals bear some similarity to the two *Drosophila* immunity pathways. In *Drosophila*, the two immune responses are activated by two different types of pathogens and are specifically suited to combat the pathogen presented. The insect antibacterial pathway is activated by pathogens that would also activate a Type 1 response in mammals. On the other hand, the insect Toll/antifungal pathway responds best to infection by fungal pathogens, which form large hyphal structures. Similar stimuli, such as helminths and certain fungi, activate a Type 2 response in mammals (Hoffmann et al. 1999; Pulendran et al. 2001). Perhaps the similarity between the two types of immune responses, found in both insects and mammals, is due to conservation of the mechanisms used to distinguish between broad classes of pathogens. Although some of the effector mechanisms are quite different, the recognition and subsequent signaling events required for activating these pathways may be very similar throughout the animal kingdom. The identification and characterization of the receptors and signaling pathways necessary for activation of innate immunity in response to a variety of pathogens, in both flies and humans, will clarify these issues.

### Mechanisms of NF- $\kappa$ B activation

Much has been learned recently about innate immunity in both mammals and flies, and knowledge from these two systems has been highly complementary (see Table 1). The most significant common feature of innate im-



**Table 1.** Comparison of innate immune signaling proteins in *Drosophila* and mammals

	<i>Drosophila</i>	Mammals
<b>Pattern recognition receptors</b>		
Non-signaling	GNBP (3)	CD14, MD-2
LPS	?	TLR4
PG, LP, zymosan	?	TLR2
Flagellin	?	TLR5
Bacterial DNA	?	TLR9
Orphan Receptors	18 wheeler, dTLR3-9	Other TLRs
<b>Cytokines and receptors</b>		
TNF $\alpha$ /TNFR	TNF-like/TNFR-like <sup>a</sup>	TNF $\alpha$ /TNFR1&2
Spätzle/Toll	Spätzle/Toll	?
IL-1 $\beta$ /IL-1R-related	None	IL-1 $\beta$ /IL-1R (8)
<b>Intracellular signaling components</b>		
Adapters	Tube, dMyD88	MyD88, Tollip
Pelle-like Kinases	Pelle	IRAK (4)
TRAF	dTRAF1-3	TRAF1-6
MAP3K	dTAK1, dMEKK	TAK1, TAB1&2, NIK, MEKK1-3
Atypical PKC	DaPKC	PKC $\zeta$ and $\lambda$ , p62
RIP-like Proteins	IMD	RIP
<b>NF-<math>\kappa</math>B components</b>		
I $\kappa$ Bs	Cactus	I $\kappa$ B $\alpha$ , $\beta$ , $\gamma$ , $\epsilon$
I $\kappa$ B Kinases		
IKK $\alpha$ / $\beta$	DMIKK $\beta$ ( <i>ird5</i> )	IKK $\alpha$ , $\beta$
IKK $\gamma$	DmIKK $\gamma$ ( <i>kenny</i> )	IKK $\gamma$ /NEMO/IKKAP
IKK $\epsilon$ -like	DmIKK $\epsilon$ /dIK2	IKK $\epsilon$ /i, TBK/NAK/T2K
NF- $\kappa$ B precursors and products	Relish	p105, p100
NK- $\kappa$ B subunits	Relish N & C	p50, p52
	Dorsal, Dif	RelA/p65, RelB, c-Rel

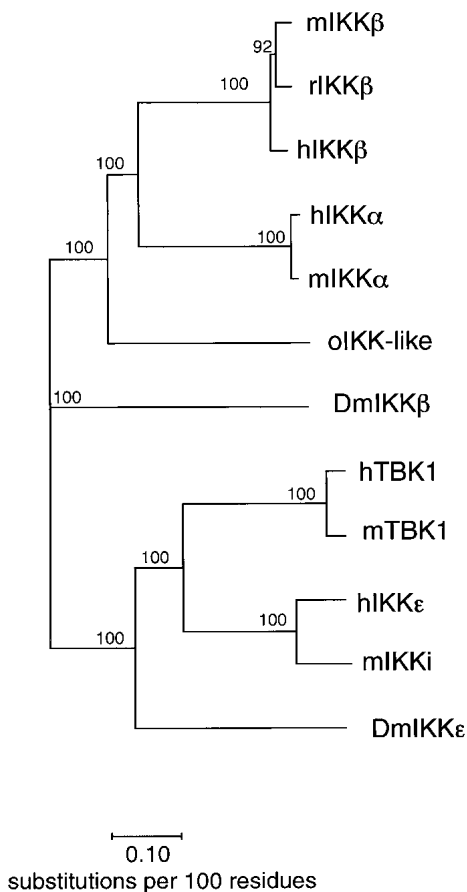
Both known and putative innate immune signaling proteins are categorized into pattern recognition receptors, cytokines and their receptors, intracellular signaling components, and NF- $\kappa$ B related kinases and transcription factors. The number of orthologs per genome are indicated in parentheses.

<sup>a</sup>The *Drosophila* TNFR-like predicted gene contains a TNFR-like cysteine-rich extracellular domain but does not include a TNFR-related intracellular domain.

munity throughout the animal kingdom is the central role of the NF- $\kappa$ B/Rel family of transcriptional activator proteins (Karin and Ben-Neriah 2000). In unstimulated cells NF- $\kappa$ B/Rel family proteins exist as hetero- or homodimeric proteins that are sequestered in the cytoplasm by virtue of their association with a member of the I $\kappa$ B family of inhibitor proteins. An astonishing number of extracellular signals can trigger distinct signal transduction pathways, each of which culminates in the destruction of I $\kappa$ B proteins. These signal transduction pathways lead to the activation of the I $\kappa$ B kinase, or IKK (for review, see Karin and Ben-Neriah 2000), and the subsequent phosphorylation of serine residues within the N-terminal destruction box of I $\kappa$ B proteins (i.e., S32 and S36 of I $\kappa$ B $\alpha$ ) (Ghosh et al. 1998). Phospho-I $\kappa$ B is then recognized by the  $\beta$ -TrCP-containing SCF ubiquitin ligase complex, leading to its ubiquitination and degradation by the proteasome (Karin and Ben-Neriah 2000). Interestingly, the active  $\beta$ -TrCP-SCF complex that functions in NF- $\kappa$ B signaling is modified by the ubiquitin-like protein Nedd8 (Read et al. 2000). The degradation of I $\kappa$ B unmasks the nuclear localization signal of the NF- $\kappa$ B/Rel family protein, leading to its nuclear translocation and binding to enhancers or promoters of target genes.

The fly I $\kappa$ B protein is known as Cactus, and by analogy with I $\kappa$ B $\alpha$  regulation, Cactus degradation is thought to be controlled by signal-dependent serine phosphorylation. However, the exact sequences required for Cactus degradation have not been definitively established (Bergmann et al. 1996; Reach et al. 1996). Moreover, the kinase responsible for signal-dependent Cactus phosphorylation has not been identified. Two IKK-related kinases are encoded in the *Drosophila* genome, although neither has been shown to be required for the Toll-induced Cactus phosphorylation and degradation (see below for more details) (Fig. 4). Like I $\kappa$ B, the ubiquitin/proteasome pathway is required for signal-dependent Cactus degradation. Mutants in *slimb*, the *Drosophila*  $\beta$ -TrCP homolog, exhibit defects in dorsoventral patterning (Spencer et al. 1999), and Cactus degradation can be blocked by proteasome inhibitors (N. Pandey, N. Silverman and T. Maniatis, unpubl.). Thus, it appears that the mechanisms involved in the activation of the *Drosophila* Dorsal and Dif proteins during early development and antifungal immunity are highly similar to those required for the activation of NF- $\kappa$ B in mammals.

NF- $\kappa$ B/Rel family proteins can also be sequestered in the cytoplasm as large precursors (the mammalian p105 and p100 proteins and the *Drosophila* Relish protein).



**Figure 4.** Phylogenetic comparison of IKK-related kinases. Phylogram of a branch-and-bound search using bootstrap analysis of aligned IKK sequences. Gaps were removed with Lineup; data were processed using PAUPDisplay and visualized as a midpoint rooted phylogram. Numbers indicate occurrence of nodes (out of 100 reiterations) during bootstrap analysis. Two subgroups of IKK are clearly distinguishable. One contains the IKK $\alpha$  and IKK $\beta$  genes (and a related protein from the oyster, *Crassostrea gigas*); the other clade contains the IKK $\epsilon$ -related genes. The *Drosophila* IKKs include DmIKK $\epsilon$ , clearly a member of the IKK $\epsilon$  subgroup, and DmIKK $\beta$  which is not truly in either subgroup, although within the kinase domain it is most similar to IKK $\beta$ . (m) Mouse; (r) rat; (h) human; (o) oyster; (Dm) *Drosophila melanogaster*.

The N termini of these proteins contain the DNA binding and dimerization domains (the Rel homology domain), whereas the C termini contain a series of ankyrin repeat sequences similar to those present in I $\kappa$ B proteins. Thus, these precursor proteins are sequestered in the cytoplasm by virtue of their covalent attachment to an I $\kappa$ B-like inhibitory domain. The C-terminal domain can be removed by proteolysis, either constitutively or, at least in the case of p100 and Relish, in response to signals (see discussion below).

#### I $\kappa$ B kinases

Key components of the NF- $\kappa$ B signaling pathways are the I $\kappa$ B kinases (IKKs) (Karin and Ben-Neriah 2000). An I $\kappa$ B

kinase capable of specifically phosphorylating serines 32 and 36 of I $\kappa$ B $\alpha$  was originally identified as a high-molecular-weight complex (~700kD) (Chen et al. 1996; Lee et al. 1998). Subsequently, two catalytic subunits (IKK $\alpha$ /1 and IKK $\beta$ /2) and a structural subunit of this complex (IKK $\gamma$ /NEMO/IKKAP) were identified and cloned (Karin and Ben-Neriah 2000). More recently, two related kinases known as IKK $\epsilon$ /IKKi and TBK/NAK/T2K were characterized and found to be in complexes distinct from that of IKK $\alpha$ / $\beta$ / $\gamma$  (Fig. 4) (Peters and Maniatis 2001).

The IKK $\alpha$ / $\beta$ / $\gamma$  complex can be activated by a variety of stimuli, including inducers of the innate immune response, such as infection by virus or treatment with LPS (O'Connell et al. 1998; Chu et al. 1999; Fischer et al. 1999; Hawiger et al. 1999). Activation of the complex involves the phosphorylation of two serine residues located in the "activation loop" within the kinase domain of IKK $\alpha$  or IKK $\beta$ . Certain MAP3 kinases (MEKK1, MEKK2, MEKK3, and NIK) are capable of phosphorylating these serines in vitro, and activating NF- $\kappa$ B in transfection experiments (Karin and Ben-Neriah 2000). In addition, dominant negative mutants of either MEKK1 or NIK kinase can inhibit NF- $\kappa$ B activation in response to certain inducers. However, it has been difficult to establish definitively a role in NF- $\kappa$ B activation for either of these kinases under physiological conditions. For example, IKK activation and I $\kappa$ B degradation occur normally in embryonic fibroblasts from NIK knockout mice (Yin et al. 2001). Curiously, although the NIK $^{-/-}$  cells display normal NF- $\kappa$ B DNA-binding activity in response to numerous stimuli, they exhibit weak activation of NF- $\kappa$ B-dependent genes specifically in response to lymphotoxin- $\beta$  receptor (LT $\beta$ R) signaling. This can now be explained by the observation that NIK is an IKK $\alpha$  kinase required specifically for signal-dependent p100 processing (see below for more details) (Matsushima et al. 2001; Xiao et al. 2001). Thus, the phosphorylation of IKK $\beta$  by NIK observed in vitro does not appear to be physiologically significant.

Similarly, although MEKK1 can phosphorylate both IKK $\alpha$  and IKK $\beta$  in vitro, *mekk1* $^{-/-}$  mouse embryonic fibroblasts (MEFs) display normal NF- $\kappa$ B activation in response to TNF $\alpha$  (Yujiri et al. 2000). By contrast, MEKK3, which can also phosphorylate IKK $\beta$  in vitro, is required for TNF $\alpha$ -induced NF- $\kappa$ B activation. *mekk3* $^{-/-}$  MEFs exhibit a greatly decreased level of IKK activation, I $\kappa$ B degradation, and NF- $\kappa$ B activation in response to TNF $\alpha$  (Yang et al. 2001).

Alternatively, in some cases the IKK complex may be activated simply by virtue of its recruitment to the receptor complex at the cell membrane (Inohara et al. 2000). This idea is based in part on the observation that IKK $\gamma$ , which is essential for IKK activation, specifically interacts with the TNF receptor-associated protein RIP (Devin et al. 2000; Poyet et al. 2000; Zhang et al. 2000), and that the IKK complex is recruited to the activated receptor via the IKK $\gamma$ -RIP interaction. In addition, IKK $\alpha$  and IKK $\beta$  are both capable of autophosphorylation and cross-phosphorylation of their activation domains (Delhase et al. 1999; O'Mahony et al. 2000). Finally, forced

multimerization of the IKK $\alpha$ / $\beta$ / $\gamma$  complex can lead to its activation (Poyet et al. 2000). Thus, the recruitment of the kinase complex to the intracellular domains of various receptors, leading to its increased local concentration, may, under some circumstances, be sufficient to activate the kinase.

Whatever the mechanisms of activation, it is clear that the IKK $\alpha$ / $\beta$ / $\gamma$  complex is required for NF- $\kappa$ B activation in response to most NF- $\kappa$ B inducers. In fact, *ikk $\beta$ <sup>-/-</sup>* mice display fetal liver apoptosis, much like the NF- $\kappa$ B p65 subunit knockout mice (Q. Li et al. 1999; Z. Li et al. 1999; Tanaka et al. 1999). Moreover, similar to the p65 knockout mice (Rosenfeld et al. 2000; Alcamo et al. 2001), when the *ikk $\beta$ <sup>-/-</sup>* mice are crossed into a *tnfr1<sup>-/-</sup>* mouse the liver apoptosis is suppressed. Thus, liver apoptosis in both cases is TNF-dependent. Finally, *ikk $\beta$ <sup>-/-</sup>* MEFs do not degrade I $\kappa$ B $\alpha$  or activate NF- $\kappa$ B in response to various stimuli including TNF $\alpha$  and LPS. Interestingly IKK $\alpha$  also plays a role in NF- $\kappa$ B activation, as *ikk $\beta$ <sup>-/-</sup>* cells still have residual IKK activity and NF- $\kappa$ B transcriptional response. However, IKK $\alpha$ / $\beta$  double knockout cells have no NF- $\kappa$ B response (Li et al. 2000). Based on the observation that IKK $\alpha$  can activate IKK $\beta$ , it was proposed that IKK $\alpha$  actually functions as an IKK-kinase, phosphorylating IKK $\beta$  (O'Mahony et al. 2000). However, this proposal is based primarily on overexpression experiments and is not supported by any of the phenotypes observed in IKK $\alpha$ <sup>-/-</sup> mice.

Remarkably, IKK $\alpha$ , but not IKK $\beta$ , appears to play a role in keratinocyte differentiation and proliferation (Hu et al. 1999; Takeda et al. 1999). *ikk $\alpha$ <sup>-/-</sup>* mice have a skin abnormality caused by the continued proliferation of stem cells and the lack of keratinocyte differentiation. Although the target of IKK $\alpha$  in keratinocyte differentiation is unknown, it does not appear to be in the NF- $\kappa$ B pathway, as none of the NF- $\kappa$ B knockout mice display a similar skin phenotype. In addition, neither the IKK $\alpha$  kinase activity nor the NF- $\kappa$ B pathway are required for the normal keratinocyte differentiation (Hu et al. 2001). Rather, it appears that IKK $\alpha$  controls production of a soluble factor that induces keratinocyte differentiation.

Another difference between IKK $\alpha$  and IKK $\beta$ , which is discussed in more detail below, is that IKK $\alpha$ , but not IKK $\beta$ , is required for the phosphorylation-dependent proteolytic processing of the p100 precursor of p52, which plays a critical role in B cell maturation and formation of secondary lymphoid organs (Senftleben et al. 2001).

The phenotype of IKK $\gamma$ -deficient mice is consistent with the phenotype of IKK $\gamma$ -deficient cells (Yamaoka et al. 1998). These mice display fetal liver apoptosis similar to that observed in IKK $\beta$  and p65 knockout mice. Furthermore, *ikk $\gamma$ <sup>-/-</sup>* MEFs are sensitive to TNF and cannot activate NF- $\kappa$ B in response to TNF $\alpha$  or LPS (Rudolph et al. 2000). Interestingly, the human disease incontinentia pigmenti (IP) appears to be caused by IKK $\gamma$  mutations (Smahi et al. 2000). This is a dominant X-linked disease with perinatal lethality in males. In heterozygous females, cells expressing only the mutant IKK $\gamma$  gene (because of X chromosome inactivation) die shortly after birth. Cells from patients, or mice, with homozygous

IKK $\gamma$  mutations show no detectable IKK $\gamma$  protein, they do not activate NF- $\kappa$ B in response to TNF $\alpha$ , and they are more sensitive to TNF-induced cell death (Makris et al. 2000; Schmidt-Supprian et al. 2000; Smahi et al. 2000).

The role of the other two IKK-related kinases, IKK $\epsilon$ /i and TBK1/T2K/NAK, is less clear. Transfection experiments with wild type and dominant negative mutants of recombinant TBK1 suggested that TBK1 functions in the NF- $\kappa$ B pathway through TANK and TRAF2. However, the signaling pathways that rely on the TBK1-TANK-TRAF complex remain to be identified. The same kinase, termed NAK (NF- $\kappa$ B Activating Kinase), was proposed to function upstream of the IKK complex and to activate IKK $\beta$  by direct phosphorylation of the activation loop serine residues, during PMA and growth factor-mediated signaling (for review, see Peters and Maniatis 2001).

On the other hand, deletion of this gene in mice, referred to as T2K, suggests a role in TNF signaling. The phenotype of *t2k<sup>-/-</sup>* mice is very similar to that of the IKK $\beta$  and p65 knockouts, as they display severe TNF $\alpha$ -induced embryonic liver degeneration and apoptosis. Thus, T2K appears to be a key component in the TNF signaling pathway in liver cells. *t2k<sup>-/-</sup>* MEFs exhibit decreased activation of some, but not all, NF- $\kappa$ B responsive genes in response to TNF $\alpha$  or IL-1 $\beta$ . However, *t2k<sup>-/-</sup>* MEFs display normal induction of IKK activity, I $\kappa$ B $\alpha$  degradation, and NF- $\kappa$ B DNA binding activity (Peters and Maniatis 2001). These observations suggest that T2K functions after I $\kappa$ B $\alpha$  degradation. For example, T2K could function to directly or indirectly activate the transcriptional activity of the p65 subunit of NF- $\kappa$ B. Other possible explanations are that T2K is required for a separate TNF-inducible pathway, such as the JNK pathway, or that T2K is required for the activation of a certain subset of NF- $\kappa$ B dimers. Those genes whose transcription requires this particular dimer would thus be most affected by the T2K deletion.

In vitro, both TBK1/T2K/NAK and IKK $\epsilon$  can phosphorylate the N-terminal regulatory domain of I $\kappa$ B $\alpha$ . This phosphorylation occurs only on serine 36, even though residues 32 and 36 must be modified to induce I $\kappa$ B $\alpha$  degradation. However, in response to PMA stimulation, IKK $\epsilon$  associates with an unidentified kinase activity that phosphorylates serines 32 and 36 of I $\kappa$ B $\alpha$ . Although the identity of the IKK $\epsilon$ -associated IKK is unknown, it was demonstrated that it is neither IKK $\alpha$  nor IKK $\beta$ . In Jurkat cells expression of dominant negative IKK $\epsilon$  blocks PMA- and TCR-mediated, but not TNF $\alpha$ -induced, NF- $\kappa$ B activation. These data argue that in mammals an alternate IKK complex exists that is responsible for I $\kappa$ B $\alpha$  phosphorylation and degradation in response to a certain subset of stimuli, for example, T-cell activation (Peters and Maniatis 2001).

As mentioned above, *Drosophila* has two IKK related genes. One, known as DmIKK $\epsilon$ , is a member of the IKK $\epsilon$ /TBK subfamily of IKKs and its function remains unknown. The other *Drosophila* IKK relative is known as DmIKK $\beta$  (or DLAK) and is required for the antibacterial immune signaling pathway (Kim et al. 2000a; Silverman et al. 2000). Although DmIKK $\beta$  is most similar to hIKK $\beta$

(and thus its name), it is in a subfamily of its own, as it has little homology with either IKK $\alpha$  or IKK $\beta$  in its C-terminal half (Fig. 4). DmIKK $\beta$  was shown to be part of a high-molecular-weight *Drosophila* IKK complex that also contains DmIKK $\gamma$ , a homolog to hIKK $\gamma$ /NEMO/IK-KAP (Silverman et al. 2000). The DmIKK complex is activated by LPS treatment, directly phosphorylates Relish, and is essential for Relish activation (cleavage) and the induction of antibacterial peptide gene transcription. These observations led to the proposal that once activated by LPS, the DmIKK complex phosphorylates Relish, which is then cleaved by an unidentified protease (Fig. 2) (Silverman et al. 2000).

DmIKK $\beta$  has also been shown to phosphorylate Cactus (Kim et al. 2000a) and the sites of phosphorylation have been mapped to the N-terminal regulatory domain of Cactus (Silverman et al. 2000). However, the significance of this modification is not clear. Mutations in the DmIKK $\beta$  or DmIKK $\gamma$  genes, *ird5* or *kenny*, respectively, cause defects in antifungal immunity, but have relatively little effect on antifungal immunity and dorsoventral patterning (Rutschmann et al. 2000b; Lu et al. 2001). Curiously, Lu et al. report that the *ird5* mutants have a slight dorsoventral phenotype showing dorsalization in 0.5% of embryos laid by *ird5* mothers. Thus, it is possible that DmIKK $\beta$  plays a role in the Toll pathway in a redundant manner with other unidentified kinases.

### Proteolytic processing of NF- $\kappa$ B/Rel precursor proteins

#### *Mammalian p100 and p105 proteins*

The p50 and p52 subunits of NF- $\kappa$ B are generated by proteolytic processing of p105 and p100 precursors, respectively. In both cases the rel homology domain is located at the N terminus of the precursor, whereas the C-terminal I $\kappa$ B-like domain functions as a covalently attached I $\kappa$ B inhibitor protein. Both precursors are processed in vivo and in vitro by a mechanism requiring ubiquitination and partial degradation by the 26S proteasome (Chen and Maniatis 1998; Karin and Ben-Neriah 2000). The regulation of p105 and p100 processing appears to play important biological roles, as mice lacking the precursor protein but able to express the mature protein display specific defects in the immune and inflammatory pathways (Ishikawa et al. 1997, 1998).

Studies of the sequence requirements for p105 processing revealed that p105 molecules containing ~110 amino acids beyond the C terminus of p50 are accurately processed, and a glycine-rich region (GRR), located just upstream from the p50 C terminus, is essential for cleavage (Lin and Ghosh 1996). Although the GRR was initially thought to be both necessary and sufficient for p50 generation (Lin and Ghosh 1996), subsequent studies revealed that additional sequences containing ubiquitination sites downstream from the GRR are also necessary for p105 processing (Fig. 5) (Orian et al. 1999).

A unique feature of p105 processing is the limited degradation by the proteasome. It is possible that this is accomplished by a mechanism in which the C terminus

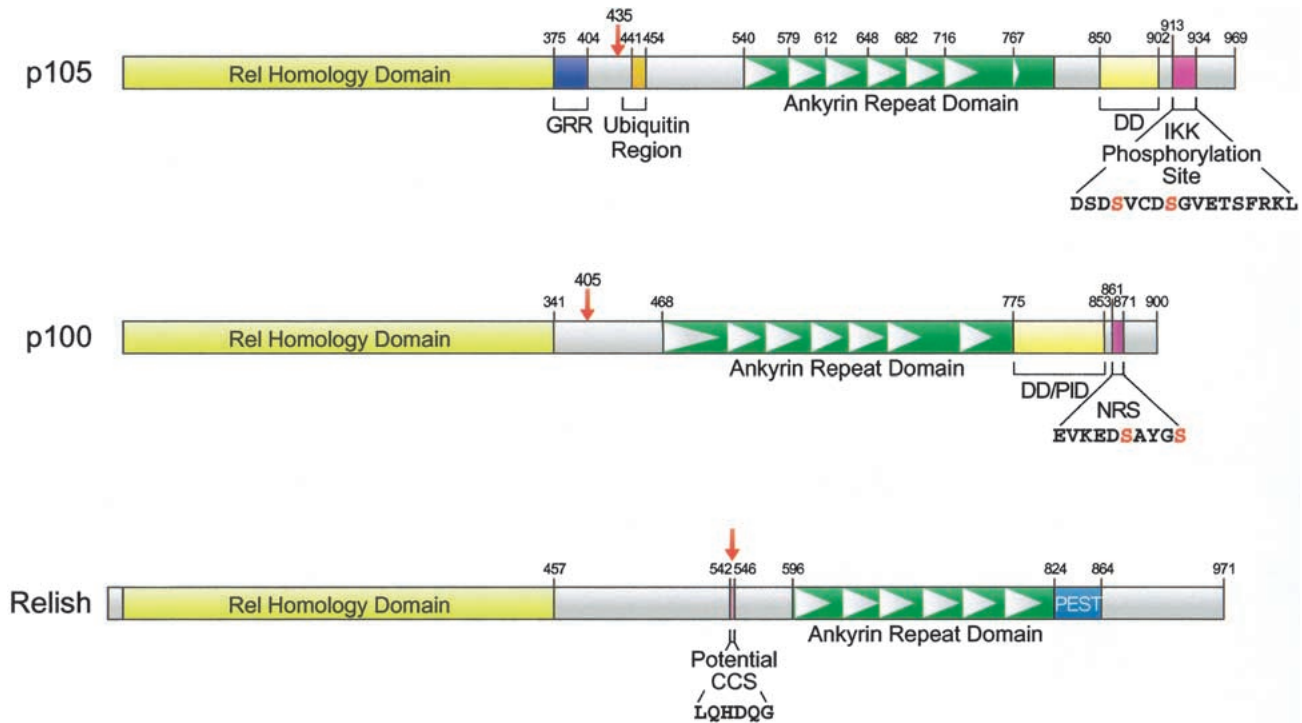
of the precursor protein enters the proteasome and is degraded processively. In this model the GRR functions as a stop signal and protects p50 from degradation (Orian et al. 1999). This model is further supported by the recent observation that ATP-dependent proteases, including the proteasome, degrade substrates processively from a degradation signal (Lee et al. 2001). However, more studies are required to prove this model. The detailed mechanism by which NF- $\kappa$ B precursors are processed by the proteasome is controversial. A number of studies have clearly demonstrated a precursor/product relationship for both proteins (Chen and Maniatis 1998; Karin and Ben-Neriah 2000). However, it has also been suggested that p105 and p100 are not the precursors of p50 and p52, but function primarily as I $\kappa$ Bs in the cytoplasm. Instead, p50 and p52 were proposed to be generated by a cotranslational processing mechanism (Lin et al. 1998; Heusch et al. 1999; Lin et al. 2000).

Regardless of the processing mechanism, the most important question is whether it is regulated. Regulated processing is difficult to demonstrate for p50 because expression of the p105 gene is itself regulated by NF- $\kappa$ B. Thus, increases in p50 could be due to either increased processing or increased p105 expression. An important insight into the mechanism of p105 processing/degradation was provided by the observation that the C terminus of p105 is phosphorylated in response to signals that activate NF- $\kappa$ B (Heissmeyer et al. 1999; Orian et al. 2000). Several different kinases have been shown to interact with and phosphorylate p105, including TPL-2 (Belich et al. 1999) and the IKKs (Heissmeyer et al. 1999; Orian et al. 2000). However, recent experiments with TPL-2 knockout mice have shown that TPL-2 is not required for LPS-dependent activation of NF- $\kappa$ B (Dumitru et al. 2000). Thus, the connection between TPL-2 and p105 remains in question.

IKK $\alpha$  and IKK $\beta$  interact with and directly phosphorylate the C terminus of p105 (Heissmeyer et al. 1999, 2001). The phosphorylated serine residues are found in a conserved DSXXXDS destruction box motif, which is recognized by the same SCF- $\beta$ TrCP ubiquitin ligase complex that recognizes the destruction box in I $\kappa$ B $\alpha$ . Similar conclusions were reached in an independent study in which the phosphorylation and ubiquitination of p105 were stimulated by cotransfection p105 and constitutively active IKK $\beta$  (Orian et al. 2000). However, the consequence of this phosphorylation is controversial. Heissmeyer et al. (1999) concluded that the signal-dependent phosphorylation of p105 results in its degradation rather than processing. By contrast, Orian et al. (2000) argue that the phosphorylation of p105 by IKK $\beta$  can lead to processing (Ciechanover et al. 2001). Thus, in spite of considerable effort, there is as yet no definitive demonstration of a signal-dependent induction of p105 processing.

A clearer picture has emerged for the regulated processing of p100 (Xiao et al. 2001). This study also sheds light on the role of the MAP3 kinase NIK in the NF- $\kappa$ B signaling pathway. Relatively little p52 is produced in most cell types, even though p100 is present, so it ap-





**Figure 5.** NF- $\kappa$ B precursor proteins from mammals and *Drosophila*. The mammalian precursor proteins, p100 and p105, are processed in a ubiquitin–proteasome-dependent manner. Approximate cleavage sites are marked with orange arrows. p105 processing (and/or degradation) is controlled by two regions, the constitutive central ubiquitin region and the inducible C-terminal IKK $\beta$ /TrCP-dependent phosphorylation and ubiquitination element. p100 processing is stimulated by NIK in an IKK $\alpha$ -dependent mechanism. Response to NIK requires the NRS, a probable IKK $\alpha$  phosphorylation site. Constitutive processing of p100 is inhibited by the processing inhibitory domain (PID), which colocalizes with a predicted death domain (DD). Relish cleavage is stimulated by LPS treatment and requires caspase proteases, not the proteasome. A potential caspase cleavage site (CCS) is found in the linker domain of Relish.

pears that p100 processing is tightly regulated. The p100 (*nfkb2*) knockout results in defects in B cell function and abnormalities in peripheral lymphoid organs. Remarkably, a similar phenotype is also observed in *alymploplasia* (*aly*) mice, which carry a mutation in the gene encoding NIK (Shinkura et al. 1999). Because of this similarity, Sun and coworkers systemically analyzed the role of NIK in p100 activation (Xiao et al. 2001). They found that processing of transfected p100 increases dramatically when active NIK is cotransfected and that this increase is enhanced by cotransfection of the LT $\beta$ R. Moreover, this processing was not observed in splenocytes from the *aly* mouse. In contrast to earlier studies of constitutively processed p100 (Heusch et al. 1999), a clear p100 precursor/p52 product relationship was observed in pulse chase experiments. Thus, at least in the regulated processing of p100, p52 is generated by a NIK-dependent post-translational processing mechanism.

Although NIK was proposed to be the direct upstream kinase for p100 (Xiao et al. 2001), more recent studies have shown that NIK functions in p100 processing by activating IKK $\alpha$ . Specifically, NIK-induced p100 processing was shown to require IKK $\alpha$ , and recombinant IKK $\alpha$  was found to be a more efficient p100 kinase than NIK (Senftleben et al. 2001). Xiao et al. (2001) also demonstrated that phosphorylation of the C terminus of p100

leads to its polyubiquitination. Thus, it seems likely that LT $\beta$ R signaling activates NIK, which in turn activates IKK $\alpha$ , which then phosphorylates p100, leading to its ubiquitination and processing by the proteasome. This conclusion is consistent with the observation that *nik*<sup>-/-</sup> and *ikk $\alpha$* <sup>-/-</sup> cells do not respond to LT $\beta$ R activation (Matsushima et al. 2001; Yin et al. 2001). Thus, p100 provides a clear example of signal-dependent processing of NF- $\kappa$ B precursors.

#### *Drosophila Relish protein*

Like p105 and p100, Relish is a bipartite protein with an N-terminal NF- $\kappa$ B-like Rel homology domain and a C-terminal I $\kappa$ B-like ankyrin repeat domain (Dushay et al. 1996). In unstimulated cells the Relish C-terminal I $\kappa$ B module sequesters its own N-terminal NF- $\kappa$ B module in the cytoplasm. Upon activation of the antibacterial signaling pathway, Relish is proteolytically cleaved and the N-terminal NF- $\kappa$ B module translocates into the nucleus, while the stable C-terminus remains in the cytoplasm. Thus, the regulation of Relish is unique among the NF- $\kappa$ B precursor proteins. Whereas processing of p100 and p105 is a proteasome-dependent event that does not leave a stable C-terminal domain, Relish cleavage is not mediated by the proteasome and results in a stable C

terminus. In fact, Relish processing may be controlled by a caspase protease and is stimulated by the *Drosophila* IKK complex (see below). It is interesting to speculate that p105 and p100 may be processed by a similar mechanism in response to inducers that have yet to be identified.

### Rel phosphorylation

Another level of regulation of NF- $\kappa$ B/Rel activity in both mammals and *Drosophila* is the phosphorylation of Rel proteins. A number of studies have shown that the signal-dependent activation of NF- $\kappa$ B requires both I $\kappa$ B $\alpha$  degradation and Rel protein phosphorylation, and these two steps can be uncoupled (Schmitz et al. 2001). For example, in the absence of Cactus, the nuclear translocation of Dorsal remains signal dependent (Bergmann et al. 1996; Drier et al. 2000), and this correlates with the signal-dependent phosphorylation of Dorsal (Drier et al. 1999). Although multiple serine residues are phosphorylated, when serine 317 is substituted by alanine a significant embryonic phenotype is observed, and Dorsal does not translocate to the nucleus.

In mammals the NF- $\kappa$ B p65 protein has a protein kinase A phosphorylation site on serine 276, and phosphorylation of this residue is required for efficient binding to the transcriptional activator protein CBP. According to a model proposed by Ghosh and colleagues, PKA is associated with the I $\kappa$ B $\alpha$ /NF- $\kappa$ B complex in the cytoplasm in an inactive form, and signal-dependent degradation of I $\kappa$ B $\alpha$  allows PKA to phosphorylate serine 276 (Zhong et al. 1997). Phosphorylation of S276 induces a conformational change in p65, exposing a phosphorylation-independent CBP interaction site and creating a phosphorylation-dependent interaction with the Kix domain of CBP (Zhong et al. 1998).

The TNF $\alpha$ -dependent phosphorylation of serine 529 has also been shown to increase the transcriptional activity of p65, and this stimulation is not at the level of nuclear translocation or DNA binding. Recently, casein kinase II was implicated in the TNF $\alpha$ -dependent phosphorylation of serine 529 (Wang and Baldwin 1998; Wang et al. 2000). Similar to the situation with PKA, I $\kappa$ B $\alpha$  protects p65 from phosphorylation by constitutively active CKII, but signal-dependent degradation of I $\kappa$ B $\alpha$  exposes the p65 phosphorylation site to CKII activity. CKII was shown to associate with p65 in vivo, and this association decreased upon TNF $\alpha$  induction. Moreover, CKII phosphorylates p65 at serine 529 in vitro, and inhibitors of CKII block phosphorylation and transcriptional activation in vivo (Wang et al. 2000). Thus, once released from I $\kappa$ B $\alpha$ , at least two kinases, PKA and CKII, phosphorylate p65, at different serine residues, to increase its transcriptional activity.

Other kinases have also been shown to act on p65. For example, IL-1 treatment has been shown to induce the phosphorylation of p65, and this phosphorylation requires phosphatidylinositol-3 kinase and Akt (Sizemore et al. 1999). Also, Akt has been implicated in Ras-induced NF- $\kappa$ B activation (Madrid et al. 2000). In these

studies, Akt signaling required IKK $\beta$  and serines 529 and 536 of p65 (Madrid et al. 2001). Similarly, overexpression of IKK $\beta$  led to the phosphorylation of p65 at serine 536 (Sakurai et al. 1999). Serine residues 529 and 536 were shown to be required for the activation of the p65 fusion proteins by activated Akt or treatment with IL-1 $\beta$  (Madrid et al. 2001). Thus, activated PI3K and Akt appear to induce NF- $\kappa$ B-dependent transcription by activating p65 rather than by promoting the degradation of I $\kappa$ B $\alpha$  and nuclear translocation of NF- $\kappa$ B.

As mentioned above, in mouse embryonic fibroblasts lacking functional T2K, I $\kappa$ B $\alpha$  phosphorylation and degradation occur in response to TNF $\alpha$ , but NF- $\kappa$ B reporter genes are not activated, suggesting the possibility that phosphorylation of Rel proteins by T2K is required for transcriptional activity. In addition, inactivation of the mouse gene encoding the glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) has no effect on the degradation of I $\kappa$ B $\alpha$  and nuclear translocation of p65 in response to TNF $\alpha$ , yet causes decrease in NF- $\kappa$ B DNA-binding activity and a defect in NF- $\kappa$ B transcriptional activity (Hoefflich et al. 2000). Thus, GSK-3 $\beta$  and T2K appear to act downstream of I $\kappa$ B $\alpha$  degradation, possibly at the level of Rel protein activation. Another possible explanation for the NF- $\kappa$ B phenotype observed in the GSK and T2K knockout MEFs is that these kinases may play a role in the activation of only a certain subset of Rel dimers. For example, they could promote the processing of Rel protein precursors (p100 and p105), similar to the function of NIK in p100 processing (Xiao et al. 2001; Yin et al. 2001). In any case, there is ample evidence to support the idea that p65 phosphorylation is critical for full NF- $\kappa$ B activation, and this activation can be uncoupled functionally from I $\kappa$ B $\alpha$  phosphorylation and degradation.

Inducible phosphorylation of Rel proteins appears to function at many different levels, including inducing conformational changes in the activation domain, increasing DNA binding activity, and promoting association with transcriptional coactivator proteins such as CBP/p300 (Schmitz et al. 2001). The overall function appears to be in the integration of signaling pathways to activate distinct Rel proteins in response to different signals.

### Toll-like receptors

The TLRs are characterized by an extracellular domain containing a number of leucine-rich repeats (LRRs), a single pass transmembrane domain, and an intracellular signaling domain, referred to as a TIR (Toll/IL-1R/Resistance) domain (Wilson et al. 1997; Zhang and Ghosh 2001). The TIR domain is common to the TLRs, the IL-1R family, and a number of plant genes that are required for host defense signaling. The IL-1R family is distinct from the TLR family because of a dissimilar extracellular domain, which consists of Ig domains. In all these receptors, the TIR domain mediates activation of intracellular signaling pathways. At least 10 TLRs are found in the mammalian genome (Aravind et al. 2001; Zhang and

Ghosh 2001), whereas *Drosophila* encodes 9 (Tauszig et al. 2000).

In mammals, TLR4 is required for LPS-mediated signaling and is believed to directly recognize LPS. LPS is a potent activator of innate immunity, and excessive exposure to LPS, or endotoxin, causes serious pathological effects (Beutler 2000). The mechanism of LPS signaling remained a mystery until the discovery of the human TLRs and the finding that hTLR4 is necessary for LPS signaling. The clearest demonstration of the role of hTLR4 in LPS signaling was provided by the positional cloning of *lps*, a mutation that causes hyporesponsiveness to LPS in mice. *lps* mutants carry loss-of-function mutations in the mTLR4 gene (Poltorak et al. 1998). Furthermore, TLR4 appears to directly bind to LPS. Lipid A is the active component of LPS and deacetylated lipid A is immunostimulatory in murine but not human systems. Two groups recently demonstrated that expression of mTLR4, but not hTLR4, enabled recognition and signaling in response to deacetylated lipid A (Lien et al. 2000; Poltorak et al. 2000). In addition to TLR4, two other extracellular proteins are involved in LPS recognition. CD14, a GPI anchored protein, has long been known to bind to LPS and is required for LPS signaling (Moore et al. 2000). MD-2 interacts with extracellular domain of TLR4 and is necessary for LPS signaling (Shimazu et al. 1999; Schromm et al. 2001). Also, Ulevitch and colleagues recently demonstrated that LPS is found in close proximity to TLR4, CD14, and MD-2 (da Silva Correia et al. 2001). This argues that TLR4, CD14, and MD-2 bind to LPS, possibly in a tetrameric complex. Interestingly, CD14 and TLR4 have also been implicated in the immune recognition of proteins encoded by viral pathogens such as RSV (Kurt-Jones et al. 2000).

*Drosophila Toll*, the founding member of the TLR family, is required for both development and immunity. During early embryonic patterning, Toll is required for establishing the dorsoventral axis. Toll is activated by the spatially and temporally restricted activation of its putative ligand, Spätzle (Belvin and Anderson 1996). This is believed to be accomplished by the correspondingly restricted activation of a serine protease cascade consisting of the products of the *nudel*, *gastrulation defective*, *snake*, and *easter* genes (LeMosy et al. 1999, 2001; Dissing et al. 2001). Activation of this protease cascade is regulated by the products of the *windbeutel* and *pipe* genes. Interestingly, *pipe* is the only one of the 12 dorsal group genes expressed in a spatially restricted manner, on the ventral side of the embryo. The spatially restricted expression of *pipe* is therefore thought to be the key signal activating the entire cascade in the correct location to establish ventral cell fates (Sen et al. 1998). The *pipe* gene product is a heparin-sulfotransferase, and it has been proposed to create a heparin-modified proteoglycan that is required for local activation of the protease cascade. Activation of Toll, by Spätzle, stimulates a signaling pathway culminating in the activation of the *Drosophila* Dorsal protein and the expression of ventral specific genes such as *twist* and *snail* (Belvin and Anderson 1996).

As mentioned earlier the Toll signaling pathway, including Spätzle, is also responsible for the antifungal immunity in insects (Lemaitre et al. 1996). However, none of the genes that function upstream of Spätzle during development are necessary for the antifungal immune response. A mutation in a *Drosophila* serpin gene (serine protease inhibitor), known as *necrotic*, has been shown to cause constitutive activation of the antifungal Toll pathway (Levashina et al. 1999). Thus, it appears that a different serine protease cascade is activated by fungal infection and leads to the processing of Spätzle. The identity of these proteases and the mechanism of their activation remain unknown. Thus, in *Drosophila*, Toll is indirectly required for immune recognition of pathogens.

Analysis of the complete *Drosophila* genome sequence identified nine different Toll-related receptors: *Toll*, *18wheeler*, and *dTLR3–9* (Tauszig et al. 2000). It is possible that one or more of these *Drosophila* TLRs function as an LPS receptor, analogous to the role of TLR4 in mammals. In fact, mutants in the TLR receptor *18wheeler* have some defects in antibacterial immune signaling. Specifically, the antimicrobial peptide gene *attacin* is not fully activated in response to *Escherichia coli* infection. However, induction of the other antibacterial peptides, such as dipterin, occurs at near wild-type levels in *18wheeler* mutants. Therefore, *18w* is not strictly required for the antibacterial immune response and cannot be the sole LPS receptor (Williams et al. 1997). The possible immune function of eight *Drosophila* TLRs was investigated recently (Tauszig et al. 2000). Remarkably, potentially dominant activated versions of these TLRs did not mimic antibacterial signaling, suggesting that none of the dTLRs are the LPS receptor. One possible explanation for this result is that two dTLRs must heterodimerize to create the fully functional LPS receptor, similar to TLR2 and TLR6 in the recognition of peptidoglycan (Ozinsky et al. 2000). Studies with dominant negative receptors, and ultimately genetic experiments with TLR mutants, are necessary to definitively determine the role of the *Drosophila* TLRs in the insect antibacterial immune response. One extracellular protein involved in LPS signaling in *Drosophila* is DGNBP1 (*Drosophila* gram-negative binding protein), which was shown to bind to LPS. DGNBP1 does not have transmembrane or intracellular domain and, like CD14, is held at the membrane by a GPI anchor. Overexpression of DGNBP1 potentiates LPS signaling while antibody interference with DGNBP1 inhibits LPS signaling. This argues that DGNBP1 plays an important role in the antibacterial immune response; however it cannot be responsible for activating intracellular signaling pathways (Kim et al. 2000b).

In mammals TLR2 is thought to be directly involved in recognizing cell wall components from gram positive bacteria and spirochetes, peptidoglycans, and lipoproteins (Krutzik et al. 2001; Zhang and Ghosh 2001). Also, TLR9 is required for the recognition of bacterial DNA (Hemmi et al. 2000). Bacterial DNA is a potent immune stimulatory molecule and is specifically recognized because of its unmethylated CpG dinucleotides (Krieg



1996). Recently, TLR5 has been shown to mediate the induction of the immune response by the bacterial flagellin protein (Hayashi et al. 2001). However, the mechanisms of TLR-mediated recognition remain unclear; the interactions of TLR2, TLR5, or TLR9 with any of their putative ligands have yet to be demonstrated biochemically. Further complicating our understanding of TLR-mediated recognition, a recent paper demonstrated that TLR2, but not TLR4, is responsible for the recognition of LPS from *Porphyromonas gingivalis* bacteria, which has a slightly different structure compared to that of *E. coli*-derived LPS (Hirschfeld et al. 2001).

It will be interesting to discover if any of the mammalian TLRs have protein ligands, like *Drosophila* Toll, and if any of the *Drosophila* TLRs can directly recognize pathogen-derived molecules. An alternative model is that the Toll-like receptors recognize ligands that contain both protein and non-protein components. For example, the protein MD-2 was shown to associate with the ectodomain of TLR4, and to be required for LPS signaling in mammalian tissue culture (Shimazu et al. 1999; Schromm et al. 2001). Thus, MD-2 may be considered a "co-ligand" with LPS for hTLR4. In *Drosophila*, the biochemical requirements for Spätzle-Toll interaction have not yet been determined. It is possible that Spätzle binding to Toll requires an unidentified (non-protein) molecule. Perhaps the *pipe* heparin-sulfotransferase creates such a molecule or modifies Spätzle in a manner that is required for Toll binding. This possibility is consistent with the proposal "that the domain of *pipe* expression establishes . . . the spatial limits of binding of processed Spätzle to Toll" (Morisato 2001).

Hirschfeld et al. (2001) also demonstrated that signaling by the mammalian TLR2 and TLR4 lead to the activation of overlapping but non-identical genes. For example, TLR2 activation causes the expression of IL-1 $\beta$ , but not IFN $\gamma$ , whereas TLR4 activation leads to the expression of both IL-1 $\beta$  and IFN $\gamma$ . This clearly demonstrates that activation of different TLRs by different types of pathogens promotes the generation of non-identical signaling pathways. These signals will then tune the immune system to activate the appropriate effector responses. It is not clear how the different TLRs can activate different sets of genes. In the studies published to date, the signaling pathways activated by various TLRs have been found to be very similar (Medzhitov et al. 1998; Yang et al. 1999). However, it is now clear that more careful analyses of the differences between these receptor-activated signaling pathways are required. Possibly, the TLR2 and TLR4 activate different NF- $\kappa$ B species, or the differences may arise from the other pathways that are activated in conjunction with NF- $\kappa$ B. In fact, a new report suggests that the TLR4 signaling, but not the TLR2 signaling, uses the p38 MAPK pathway (Rabehi et al. 2001). In this way, the appropriate response would be activated by each TLR in response to different pathogens.

The TLRs have been implicated in the cell surface recognition of many pathogen-derived substances. How-

ever, the mechanisms by which foreign substances are recognized within the cell are poorly understood. For example, virus infection is a potent activator of pro-inflammatory signaling pathways leading to the activation of NF- $\kappa$ B, JNK, and IRF proteins (Maniatis et al. 1998). Within the cell, double-stranded RNA is thought to be the pathogen-derived substance that triggers the antiviral response. A potential PRR for double-stranded RNA is the dsRNA-dependent protein kinase, PKR. However, evidence for a role of this kinase in NF- $\kappa$ B activation is controversial. Two studies have reported that *pkr*<sup>-/-</sup> MEFs are unresponsive to virus- and dsRNA-mediated induction of the IFN- $\beta$  (Yang et al. 1995; Chu et al. 1999), yet *pkr*<sup>-/-</sup> mice respond normally (Yang et al. 1995; Abraham et al. 1999). Furthermore, another group, using similar *pkr*<sup>-/-</sup> cells, reported normal dsRNA induced I $\kappa$ B degradation and NF- $\kappa$ B activation while phosphorylation of eIF-2 $\alpha$ , a known target of PKR, is not observed (Iordanov et al. 2001; B. Magun, pers. comm.). Therefore, they conclude that PKR is required for dsRNA-mediated inhibition of host cell protein synthesis but is not necessary for activation of NF- $\kappa$ B and immune-inducible genes in response to dsRNA or virus infection (Iordanov et al. 2001). At present the reason for this discrepancy is not understood.

Other possible candidates for dsRNA receptors are the NOD proteins (Bertin et al. 1999; Inohara et al. 1999; Hlaing et al. 2001; Ogura et al. 2001b). The NOD genes encode cytosolic proteins that contain a LRR domain, similar to that found in the extracellular domains of the TLRs, an ATPase domain related to APAF-1/CED4, and one or more CARD domains. At least 20 NOD-like genes have been identified in the human genome sequence (Inohara et al. 2001). Through the CARD domain, NOD1 and NOD2 can interact with RIP2/RICK and activate NF- $\kappa$ B (Bertin et al. 1999; Inohara et al. 1999; Ogura et al. 2001b). Perhaps the LRR domain of the NOD genes is involved in the recognition of dsRNA and/or other intracellular pathogen-derived substances. In fact, Nunez and colleagues have shown that NOD1 or NOD2 expression confers responsiveness to (presumably intracellular) LPS in a TLR4-independent manner (Inohara et al. 2001). Thus, the NOD genes may encode a family of PRRs that function within the cell to recognize substances released from intracellular pathogens. Interestingly, mutations in the NOD2 gene are linked to susceptibility to Crohn's disease, an inflammatory bowel disease (Hugot et al. 2001; Ogura et al. 2001a). Three independent NOD2 mutations were found associated with different Crohn's-susceptible families. One of these alleles is a one-base-pair insertion that causes premature termination, truncating the last LRR. When overexpressed both the wild-type and mutant NOD2 genes activate an NF- $\kappa$ B reporter. However, when expressed at lower levels only the wild-type, but not the mutant, NOD2 can mediate the LPS-induced activation of NF- $\kappa$ B (Ogura et al. 2001a). These findings suggest a link between Crohn's disease, which is thought to have both environmental and genetic determinants, and innate immunity.



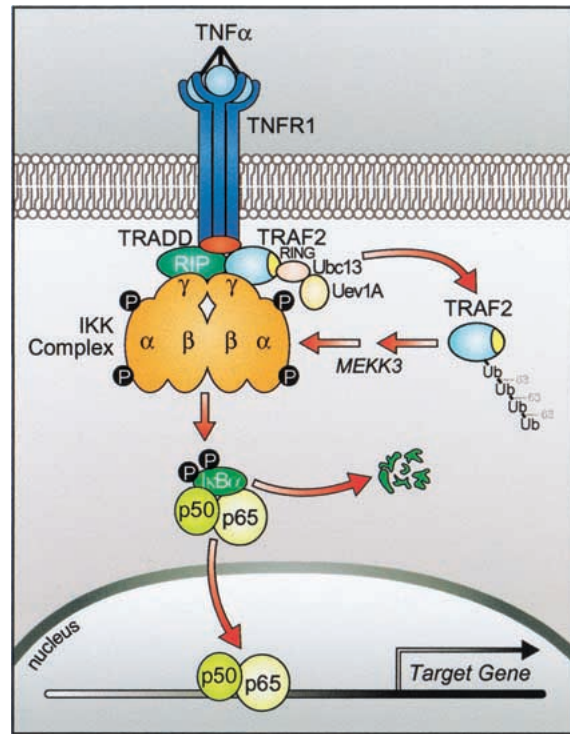
## Receptor proximal signaling events

Much current research in both the mammalian and *Drosophila* innate immunity fields is focused on the mechanisms of TLR-mediated activation of IKK complexes and NF- $\kappa$ B. The literature on this subject is confusing and sometimes contradictory, as a firm understanding of the biochemistry involved has been difficult. In the remaining section of this review, we summarize the proteins that play a role in these intracellular signaling pathways and discuss their likely function. The TNFR1 pathway is the best-studied mammalian NF- $\kappa$ B activation pathway. Although TNFR1 signaling is not activated directly by pathogens, we include it in our discussion for comparison to the TLR pathways. Also, the IL-1 $\beta$  and LPS signaling pathways are discussed together because they are very similar.

### TNF $\alpha$

TNFR1 is activated through a ligand-induced reorganization of a trimeric receptor complex that brings together intracellular death domains (Chan et al. 2000a). This conformational change may cause the dislocation of a protein, known as silencer of death domain or SODD, which normally functions to silence the TNFR1 in the absence of ligand (Jiang et al. 1999). This is followed by the assembly of a receptor proximal signaling complex, which is nucleated by the death domain binding protein TRADD. TRADD in turn recruits TRAF2 and RIP (Fig. 6) (Chan et al. 2000b). RIP is a death domain-containing kinase required for TNF $\alpha$ -mediated NF- $\kappa$ B activation (Ting et al. 1996; Kelliher et al. 1998). However, the kinase domain of RIP is dispensable for NF- $\kappa$ B activation. Instead, complementation of a RIP-deficient cell line requires the intermediate domain of RIP, which lies between the kinase domain and the death domain (Ting et al. 1996). The death domain is required for the binding of RIP to TRADD. Numerous studies with dominant negative TRAF2 proteins have shown that it is important for TNF $\alpha$  signaling. TRAF2 knockout cells exhibit reduced and delayed activation of NF- $\kappa$ B-dependent gene expression and also show deficient IKK activation (Arch et al. 1998). This intermediate phenotype observed in the *traf2*<sup>-/-</sup> cells probably reflects redundancy between TRAF2 and another TRAF gene, possibly TRAF5 (Tada et al. 2001).

Two recent studies examined the roles of TRAF2 and RIP in TNF signaling. One study, Devin et al. (2000), demonstrated that the IKK complex is activated by recruitment to the activated TNFR1 complex in a TRAF2-dependent manner. Activation of receptor-associated IKK complexes requires RIP (Devin et al. 2000). In another study, Zhang et al. (2000) also found that the IKK complex is recruited to TNFR1 in a ligand-dependent manner. In contrast to the findings of Devin et al., they show that RIP can directly interact with IKK $\gamma$ /NEMO. The data in Zhang et al. (2000) also suggest contradictory to Devin et al. that the IKK complex associated with the TNFR1 is not activated. The reason for this discrepancy is not understood.



**Figure 6.** TNFR1 signaling. In this model of TNFR1 signaling, the IKK complex is activated while associated with the receptor. The IKK complex is recruited to the receptor in a TNF $\alpha$ -dependent manner. This recruitment requires TRAF2 and may also involve the interaction between IKK $\gamma$  and RIP. TRAF2 is thought to activate the IKK complex via a ubiquitin-dependent signaling pathway. The TRAF2/ubiquitin signaling complex may lead to the activation of MEKK3, although this has yet to be demonstrated. RIP is also likely to play a role in activation of the IKK complex, possibly by interacting with MEKK3 (Yang et al. 2001). Once activated, the IKK complex phosphorylates I $\kappa$ B $\alpha$  on serines 32 and 36, leading to its proteasome-mediated degradation.

### IMD

In *Drosophila*, there is no evidence for a TNFR1-like signaling complex, although the *Drosophila* genome encodes a TNF $\alpha$ -like molecule and a receptor with a TNFR-like extracellular domain. However, this receptor does not contain a cytoplasmic domain homologous to either TNFR. The role of these TNF-like factors in *Drosophila* immunity remains unknown (Aravind et al. 2001).

Interestingly, a RIP-like molecule does play an important role in the insect antibacterial immune response. As mentioned earlier, gram-negative bacterial infection, or LPS treatment, activates a signaling cascade that culminates in the proteolytic activation of Relish. Genetic screens have identified five genes required for this signaling pathway: *ird5* (DmIKK $\beta$ ), *kenny* (DmIKK $\gamma$ ), *dTAK1*, *Dredd* (see below), and *immunodeficient* (*imd*) (Lemaitre et al. 1995; Elrod-Erickson et al. 2000; Leulier et al. 2000; Rutschmann et al. 2000b; Lu et al. 2001; Vidal et al. 2001). Recently, *imd* was cloned and found to

encode a protein with homology to the mammalian RIP protein. The *imd* protein includes a death domain most similar to that of RIP and another domain with weak similarity to the intermediate domain of RIP. Unlike RIP, *imd* does not contain a kinase domain. By analogy to mammalian RIP, IMD is predicted to function in a receptor proximal role, possibly associating with the yet-to-be identified LPS receptor. Overexpression of IMD leads to activation of the antibacterial peptide genes (S. Naitza and P. Georgel, pers. comm.).

### TLR signaling

In mammals, the first intracellular event following LPS or IL-1 $\beta$  treatment is the recruitment of the adaptor protein MyD88 to TLR4 or IL-1R complexes (Wesche et al. 1997; Medzhitov et al. 1998). MyD88 contains a TIR domain and a death domain. MyD88 interacts with TLR4 via a homotypic TIR connection, and it recruits the kinase IRAK (IL-1 receptor associated kinase) to the receptor complex via a homotypic death domain interaction. Another protein, called Tollip, may also be involved in recruiting IRAK to the IL-1R complex and in keeping the unliganded IL-1R complex from signaling (Burns et al. 2000). MyD88 is required for IL-1 and IL-18 signaling and activation of NF- $\kappa$ B (Adachi et al. 1998). *MyD88*<sup>-/-</sup> mice and macrophages were also found to be unresponsive to LPS. However, *MyD88*<sup>-/-</sup> macrophages still activate NF- $\kappa$ B DNA-binding activity in response to LPS, albeit with delayed kinetics (Kawai et al. 1999). This surprising finding suggests that LPS signaling, presumably through TLR4, requires MyD88 for its full biological response, but that macrophages do not absolutely require MyD88 to degrade I $\kappa$ B and activate NF- $\kappa$ B. The mechanism by which LPS activates NF- $\kappa$ B in a MyD88-independent manner remains unknown. One possibility is that the LPS-induced NF- $\kappa$ B response observed in MyD88-deficient cells reflects NOD signaling rather than TLR4 signaling. Another possibility is that TLR4 uses another adapter molecule in addition to MyD88. It is also not clear why MyD88 knockout mice and macrophages are unresponsive to LPS but can still activate NF- $\kappa$ B.

### Pelle/IRAK

Similar to RIP, the kinase activity of IRAK is not required for NF- $\kappa$ B activation, although IRAK is required for optimal IL-1- and LPS-induced signaling. For example, an IRAK-deficient cell line, which is defective in IL-1 signaling, can be complemented by a kinase-deficient IRAK transgene. In other experiments transfection of a kinase-dead IRAK gene leads to the activation of NF- $\kappa$ B (Knop and Martin 1999; X. Li et al. 1999; Maschera et al. 1999). In fact, IL-1-mediated NF- $\kappa$ B activation requires the N terminus (death domain and "undefined domain") and the C terminus, but not the central kinase domain of IRAK (Li et al. 2001). The conclusion that kinase activity is not required for IRAK-dependent NF- $\kappa$ B activation is tempered by the existence of four IRAK-

like genes, and the fact that IRAK2 can associate with the IL-1R complex. Thus, during LPS or IL-1 signaling, the kinase activity of IRAK could be supplied by IRAK or one of its three homologs. IRAK-deficient mice and cells (macrophages and fibroblasts) are defective in IL-1- and LPS-induced NF- $\kappa$ B activation. They exhibit weaker and slower IKK activation, NF- $\kappa$ B DNA binding activity, gene activation, and cytokine production (Kanakaraj et al. 1998; Thomas et al. 1999; Swantek et al. 2000). However, NF- $\kappa$ B is still activated, albeit more slowly and weakly, in the *irak*<sup>-/-</sup> cells. Presumably one or more of the other IRAK-like proteins can function in a partially redundant manner during IL-1- and LPS-induced signaling (Muzio et al. 1997; Wesche et al. 1999; Aravind et al. 2001). The association of IRAK with the receptor is thought to be transient. Once activated, IRAK is released from the receptor, and is thought to bind to and activate TRAF6. The IRAK/TRAF6 complex can then activate downstream targets (Cao et al. 1996; Ishida et al. 1996).

Toll signaling in *Drosophila* requires the protein Tube and the IRAK-like kinase Pelle. The receptor proximal events in *Drosophila* Toll signaling differ somewhat from those of mammalian TLR signaling. Genetic experiments demonstrated that Tube functions upstream of Pelle (Drier and Steward 1997), suggesting that Tube is the link between the receptor and the kinase. Thus, Tube can be viewed as an adapter analogous, but not homologous, to the mammalian MyD88 protein. Interestingly, a *Drosophila* MyD88-like gene is found in the *Drosophila* genomic DNA database, but its role in Toll signaling or other dTLR-mediated pathways has not been established (Aravind et al. 2001).

Activation of Toll by Spätzle leads to the recruitment of Tube to the membrane, possibly by direct interaction between Toll and Tube (Shen and Manley 1998; Towb et al. 1998). Tube interacts with Pelle through the homotypic death domain, although this binding occurs preferentially with inactive Pelle (Edwards et al. 1997; Xiao et al. 1999). Inactive Pelle has also been shown to interact with the Toll cytoplasmic domain (Shen and Manley 1998). Shen and Manley have proposed that signal-induced Pelle activation leads to the phosphorylation of Toll, Tube, and Pelle itself, allowing the dissociation of the Toll-Tube-Pelle membrane proximal complex that forms immediately upon receptor activation. The dissociation of the receptor complex would allow Pelle, and possibly Tube, to disengage from the receptor following activation, and proceed into the cytoplasm where it is required to mediate signal transduction. However, signal-dependent Pelle activation has yet to be demonstrated. Unlike IRAK-dependent signaling, the kinase activity of Pelle is required for Toll signaling (Shelton and Wasserman 1993). It has also been suggested that Tube has an additional function in the Toll pathway, acting as a co-activator with Dorsal in the nucleus (Norris and Manley 1995, 1996).

Interestingly, the Toll cytoplasmic domain and Tube have also been shown to interact with Filamin (also known as ABP-280), a component of the cytoskeleton (Edwards et al. 1997). Interestingly, the Toll-Filamin in-

teraction is reduced by missense mutations in the Toll signaling domain that prevent signaling (N. Silverman and T. Maniatis, unpubl.). Filamin may serve as scaffold protein for the assembly of the signaling components of the Toll pathway. Filamin has also been implicated in NF- $\kappa$ B activation in mammals. For example, a Filamin-deficient melanoma cell line is unresponsive to TNF or TLR activation, and complementation with Filamin expression restores some signaling to these cells (Leonardi et al. 2000).

### TRAFs

In mammals, key components in various NF- $\kappa$ B activating pathways are the TRAF proteins (Arch et al. 1998). As mentioned above TRAF2 plays a critical role in TNF signaling while TRAF6 is essential for IL-1 and LPS signal transduction. A variety of experiments have implicated TRAF proteins in the activation of NF- $\kappa$ B. For example, overexpression of TRAF2 or 6 activates NF- $\kappa$ B, and expression of dominant negative TRAF proteins blocks NF- $\kappa$ B activation (Arch et al. 1998). Moreover, TRAF6 knockout mice exhibit defects in IL-1- and LPS-induced NF- $\kappa$ B activation (Lomaga et al. 1999; Naito et al. 1999). The TRAF proteins all share a common C-terminal TRAF domain. This domain is further subdivided into two parts, the highly conserved TRAF-C domain and the coiled-coil TRAF-N domain. The TRAF domain is required for interaction with upstream factors such as IRAK, TRADD, and various receptors. In addition, the TRAF proteins contain a variable number of cysteine-rich zinc fingers N-terminal to the TRAF domain, and at their very N terminus most TRAF proteins encode a RING finger motif.

Recently, RING finger domains have been shown to play a crucial role in several ubiquitin ligase complexes implicated in a number of biological processes. For example, the RING finger-containing protein Rbx1/Roc1 is an essential component of the SCF ubiquitination complex (Deshaies 1999) and the Cbl RING finger is required for ubiquitination of receptor tyrosine kinases such as PDGFR and EGFR. In addition, the TRAF RING finger appears to be required for activation of NF- $\kappa$ B. In the case of TRAF2 and 5, the RING finger is required for NF- $\kappa$ B activation, and deletion of just the RING creates a dominant negative protein (Arch et al. 1998). By contrast, the role of the TRAF6 RING finger is less clear. For example, a number of studies have shown that NF- $\kappa$ B activation induced by overexpression of TRAF6 does not require the RING domain (e.g., Cao et al. 1996). In contrast, another study shows that mutations in the conserved cysteine or histidine residues in the RING finger abolish TRAF6-mediated NF- $\kappa$ B activation (Deng et al. 2000). In addition, most of the dominant negative TRAF6 mutants studied involve the removal of both the TRAF6 RING and Zn finger motifs (Cao et al. 1996). Moreover, forced dimerization of the RING and Zn finger domains, from either TRAF2 or TRAF6, activates signaling (Baud et al. 1999). However, Kobayashi et al. (2001) recently showed that the RING finger of TRAF6 is not absolutely required

for IL-1- and LPS-mediated NF- $\kappa$ B activation in MEFs (Kobayashi et al. 2001). Taken together these experiments suggest that TRAF6 may have both RING finger-dependent and -independent (Zn finger-dependent) mechanisms to activate NF- $\kappa$ B.

An important breakthrough in understanding the mechanisms of TRAF6-dependent activation of NF- $\kappa$ B was recently provided by Chen and colleagues. These investigators established an *in vitro* system for activating the IKK $\alpha/\beta/\gamma$  complex in response to the addition of TRAF6 (Deng et al. 2000). Biochemical fractionation studies identified two essential activities, called TRIKA 1 and 2, which are required for TRAF6-induced IKK activation. TRIKA 1 consists of a heterodimer of two ubiquitination proteins called Ubc13 and Uev1A. Strikingly, these Ubc molecules were shown to catalyze the nonclassical ubiquitination of protein substrates, forming ubiquitin chains extended on lysine 63 instead of the usual lysine 48 of ubiquitin. In the classical ubiquitin system, polyubiquitination of a substrate protein leads to its proteasome-mediated degradation. However, nonclassical ubiquitin chains, linked through lysine 63, do not lead to proteasome mediated degradation (Deng et al. 2000). Rather, this ubiquitination is thought to play a regulatory role, similar to that observed with the ubiquitin-like molecules Nedd8 and Sumo (Yeh et al. 2000). Thus, TRAF6-mediated IKK activation requires ubiquitination, but not the proteasome (Deng et al. 2000). Additional studies showed that TRAF6 functions as part of an unique E3 complex, with Ubc13 and Uev1A, and that TRAF6 itself is the target of ubiquitination (Wang et al. 2001). Remarkably, IL-1-, TNF $\alpha$ -, and TRAF2-mediated NF- $\kappa$ B activation were all blocked by expression of dominant negative, catalytically inactive Ubc13 (Deng et al. 2000). This exciting finding clearly establishes TRAF6, and the TRAF family members in general, as ubiquitin ligase components required in NF- $\kappa$ B signaling pathways. These findings also explain the puzzling observation that the partially purified unactivated IKK complex can be activated by ubiquitination *in vitro* (Chen et al. 1996).

TRIKA 2, the second activity required for TRAF6-mediated IKK activation *in vitro*, was found to consist of a complex of the MAP3K TAK1, and the associated proteins TAB1 and TAB2. Furthermore, this TAK1 complex was shown to be activated by association with K63-ubiquitinated TRAF6. Once activated TAK1 can directly phosphorylate IKK $\beta$  and MKK6, leading to the activation of both the JNK/p38 and NF- $\kappa$ B signaling pathways (Wang et al. 2001). Thus, it appears that TAK1 is the critical IKK-kinase in the IL-1 and TLR signaling pathways. This conclusion is consistent with earlier work on TAK1 showing that dominant negative TAK1 inhibits IL-1 signaling and that in response to IL-1 $\beta$  TAB2 translocates from the membrane to the cytoplasm where it associates with TAK1 (Ninomiya-Tsuji et al. 1999; Takaesu et al. 2000, 2001). In fact, Matsumoto and colleagues have previously demonstrated the IL-1 $\beta$ -dependent formation of a TAK1/TAB1/TAB2/TRAF6 complex with endogenous proteins (Takaesu et al. 2000).



This ubiquitination/TAK1 pathway may also play a critical role in *Drosophila* immunity, as the *Drosophila* TAK1 homolog, dTAK1, is critical for insect innate immunity. Lemaitre and colleagues isolated dTAK1 in a genetic screen for mutants defective in the IKK- and Relish-dependent antibacterial signaling pathway (Vidal et al. 2001). The dTAK1 protein functions downstream of IMD adaptor protein, but upstream of the DmIKK complex (Vidal et al. 2001). Furthermore, recent unpublished experiments using RNAi revealed that the *Drosophila* homologs of Ubc13 and Uev1A, *bendless* and *dUev1A*, respectively, function in the insect antibacterial signaling pathway (R. Zhou, N. Silverman, Z. Chen, and T. Maniatis, unpubl.). This suggests that a similar ubiquitin-dependent pathway may be involved in the activation of the *Drosophila* IKK complex through dTAK1. However, a clear picture for the function of *Drosophila* TRAF proteins has not been obtained. Analysis of the *Drosophila* genome sequence has identified three TRAF homologs. dTRAF1 is most similar to human TRAF4 but does not contain a RING finger (Liu et al. 1999; Zapata et al. 2000). Another, dTRAF2, is most similar to human TRAF6 and contains a RING finger. The third *Drosophila* TRAF is poorly characterized but does not appear to encode either a RING finger or zinc finger motif (Grech et al. 2000). To date none of the *Drosophila* TRAFs have been shown to be involved in either of the *Drosophila* immune signaling pathways. Thus, the RING finger-containing protein that is predicted to be involved in the ubiquitin-dependent LPS-stimulated activation of dTAK1 and subsequent DmIKK activation remains unidentified.

#### Atypical PKCs

Other potential components of the IL-1/LPS and TNF $\alpha$  signaling pathways include the atypical PKCs,  $\lambda$  and  $\zeta$ , and their associated adapter protein p62 (for review, see Moscat and Diaz-Meco 2000). Inhibition of the aPKCs or p62, by dominant negative or antisense RNA treatment, respectively, inhibits IL-1 and TNF signaling. IL-1-induced interactions have been demonstrated between endogenous TRAF6 and p62, and TNF $\alpha$  has been shown to induce an association of aPKCs and p62 with RIP and the IKK complex assembled at the activated TNFR1. Furthermore, the  $\zeta$ PKC has been shown to phosphorylate IKK $\beta$  on serines 177 and 181, within the activation loop, leading to IKK $\beta$  but not IKK $\alpha$  activation. This suggests that aPKCs may also serve as IKK-kinases (Moscat and Diaz-Meco 2000). However, these cell culture results have yet to be verified in vivo and it is not yet clear how the aPKCs function relative to other putative IKK-kinases such as MEKK3 and TAK1.

#### Caspases

Interestingly, the *Drosophila* caspase protease Dredd is also required in the antibacterial signaling pathway. In fact, *dredd* mutants are unable to cleave Relish (Stöven

et al. 2000) and to activate antibacterial peptide gene expression in response to infection (Elrod-Erickson et al. 2000; Leulier et al. 2000). In addition, the specific caspase inhibitor zVAD-fmk is a potent inhibitor of LPS-stimulated Relish cleavage and antibacterial peptide gene expression in *Drosophila* cell culture (N. Silverman and T. Maniatis, unpubl.). Dredd is an apical caspase with an extended, death effector domain (DED) containing pro-domain. Although overexpression of a Dredd isoform can cause programmed cell death in cell culture, the role of Dredd in programmed cell death in vivo remains uncertain (Chen et al. 1998; Rodriguez et al. 1999). Interestingly, the linker region of Relish, which connects the RHD and C-terminal I $\kappa$ B-like domain, contains a potential caspase cleavage site (LQHDG). These observations suggest a model in which LPS activates the *Drosophila* IKK $\beta$ / $\gamma$  complex, which in turn phosphorylates Relish. The modified Relish protein would then be recognized and cleaved by Dredd (Fig. 2). However, we have been unable to cleave phosphorylated Relish in vitro with recombinant Dredd (P. Chen, N. Silverman, T. Maniatis, J. Abrams, unpubl.). Therefore, other models must be considered. For example, it was proposed that Dredd may function similarly to caspase 1, or ICE (Fantuzzi and Dinarello 1999), to cleave and activate a cytokine-like signaling molecule that is responsible for transmitting a signal from the site of infection to the fat body (Elrod-Erickson et al. 2000).

Another possibility is suggested by the recent characterization of *Drosophila* FADD (Fas associated death domain) homolog, which contains a death domain and a DED. dFADD was shown to interact strongly and specifically with Dredd via a homotypic DED domain interaction (Hu and Yang 2000). Although the role of dFADD in immunity has yet to be demonstrated, it is reasonable to propose that dFADD may be involved in the antibacterial signaling pathway. For example, dFADD may interact with IMD via its death domain while contacting Dredd via its DED domain. In this way, Dredd may be connected to and activated by the LPS receptor. Analogous to other caspase pathways, activated Dredd could then cleave and activate an effector caspase, which would then cleave phosphorylated Relish. In this model, the DmIKK complex and Dredd would function in parallel pathways, both of which are downstream of the LPS receptor and required for signal-induced Relish activation (Fig. 2).

In mammals, the classical NF- $\kappa$ B signaling pathways are not known to require any caspase mediated cleavage events. In fact the caspase inhibitor zVAD-fmk potentiates LPS signaling in primary murine macrophages (P. Murray, N. Silverman, and T. Maniatis, unpubl.). However, the recent discovery of a human paracaspase suggests that there may be some similarity between *Drosophila* Relish activation and NF- $\kappa$ B activation in humans. By using reiterative searches and threading algorithms, comparing the similarities in the sequence and structure of mammalian caspases and a bacterial cysteine protease known as Gingpain R (Eichinger et al. 1999), Dixit and colleagues were able to identify several



new caspase related proteases in a number of genomes including one in humans. They termed these newly identified genes paracaspases (Uren et al. 2000). Paracaspases share some structural similarity to the classic caspases but are in a separate subgroup and are easily distinguishable from the classic caspases. The human paracaspase gene is associated with translocations found in patients with MALT lymphoma. This translocation fuses the BIR domains of IAP-2 to the catalytic domain of the human paracaspase gene. Expression of these IAP-paracaspase fusion genes causes the activation of NF- $\kappa$ B (Uren et al. 2000). This activation requires the proteolytic activity of the paracaspase. Also, the human paracaspase interacts with Bcl10, a well-known activator of NF- $\kappa$ B. Interestingly, Bcl10 is associated with a different translocation also found in MALT lymphoma. In fact, both paracaspase and Bcl10 translocations, t(11;18) and t(1;14), are required for MALT lymphoma pathogenesis. Another group demonstrated that forced dimerization of the paracaspase catalytic domain potentially activates NF- $\kappa$ B (Lucas et al. 2001). These findings suggest that unidentified stimuli may activate NF- $\kappa$ B through a Bcl10 and a paracaspase-dependent signaling pathway. The substrates and the substrate specificity of human paracaspase are unknown at this time.

## Conclusions

Comparison of the *Drosophila* and mammalian innate immune signaling pathways has identified both similarities and differences. For example, in both mammals and insects, the innate immune response is activated by similar pathogen-derived substances such as LPS or peptidoglycans. Intracellular signaling pathways that are activated by the Toll family of receptors lead to the activation of NF- $\kappa$ B, in both insects and mammals. In both systems NF- $\kappa$ B is required for the induction of antimicrobial effector mechanisms such as complement activation, antimicrobial peptide gene expression, and activation of phagocytosis. A major difference is that activation of the mammalian innate system leads to expression of costimulatory molecules and the subsequent activation of the adaptive immune response. Other differences include the mechanisms of immune recognition; mammals appear to use the TLRs and their LRR ectodomains to recognize a large array of microbial pathogens whereas the mechanisms used by insects remain unclear. Also, detailed analysis of the signaling pathways downstream of these receptors reveals important differences. For example, insects activate the NF- $\kappa$ B precursor Relish by IKK- and caspase-dependent endoproteolytic cleavage. On the other hand, mammalian NF- $\kappa$ Bs are activated by the proteasome-mediated destruction of I $\kappa$ B proteins, and/or the proteasome-mediated degradation of the C-terminal inhibitory domain of NF- $\kappa$ B precursors p100 and p105. In both systems, it is clear that the NF- $\kappa$ B proteins are themselves activated, probably by direct phosphorylation. Comparing the similarities and differences between mammals and insects

should continue to provide important insights into the role of NF- $\kappa$ B in the innate immune response.

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