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Signaling to NF-κB

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The transcription factor NF-κB has been the focus of intense investigation for nearly two decades. Over this period, considerable progress has been made in determining the function and regulation of NF-κB, although there are nuances in this important signaling pathway that still remain to be understood. The challenge now is to reconcile the regulatory complexity in this pathway with the complexity of responses in which NF-κB family members play important roles. In this review, we provide an overview of established NF-κB signaling pathways with focus on the current state of research into the mechanisms that regulate IKK activation and NF-κB transcriptional activity.

Inducible transcription factors regulate immediate and long-lived cellular responses necessary for organismal adaptation to environmental plasticity. Such responses are mediated to a large degree through changes in gene expression. One transcription factor that serves as a key responder to changes in the environment is NF-κB, an evolutionarily conserved signaling module that plays a critical role in many biological processes. Understanding how the transcriptional potential, activity, and selectivity of NF-κB are regulated is therefore a topic of intense investigation in numerous laboratories.

The biological system in which NF-κB plays the most important role is the immune system (for reviews, see Ghosh et al. 1998; Li and Verma 2002; Bonizzi and Karin 2004). Careful regulation of the transcriptional responses to many different stimuli is crucial to the proper functioning of the mammalian immune system. NF-κB regulates the expression of cytokines, growth factors, and effector enzymes in response to ligation of many receptors involved in immunity including T-cell receptors (TCRs) and B-cell receptors (BCRs), TNFR, CD40, BAFFR, LTβR, and the Toll/IL-1R family (for reviews, see Ghosh et al. 1998; Silverman and Maniatis 2001; Bonizzi and Karin 2004). NF-κB also regulates the expression of genes outside of the immune system and, hence, can influence multiple aspects of normal and disease physiology. Recent work has highlighted the role of NF-κB in embryonic development and in the development and physiology of tissues including mammary gland, bone, skin, and central nervous system. However, such varied biological roles for NF-κB raise the intriguing question of whether one common mechanism regulates signaling to NF-κB in all systems or whether discrete inputs create a diversity of transcriptional mechanisms that are tailored to particular tissues and organs. Understanding how NF-κB integrates multiple stimuli in multiple systems to generate a unified outcome suitable for specific situations is a challenge that faces researchers in this area. In keeping with the enormous progress that has been made in the study of NF-κB, there has been a veritable explosion of review articles that have elegantly summarized progress in different aspects of NF-κB regulation and biology (Ghosh and Karin 2002; Kucharczak et al. 2003; Ruland and Mak 2003; Ben-Neriah and Schmitz 2004; Bonizzi and Karin 2004; Chen and Greene 2004; Karin et al. 2004). Therefore, to avoid duplication, we have decided to focus in this review on a few areas of current activity. The main question that will be discussed in this review is how the different inducers activate NF-κB and the mechanisms that underlie the regulation of NF-κB transcriptional activity. The choice of these areas for discussion is, of course, idiosyncratic and we apologize for the narrow focus of this review. However, to help an uninitiated reader delve right into these areas of current research, we have provided a brief overview of the current state of knowledge about this transcription factor. We hope that interested readers will find a sufficiently comprehensive listing of the relevant literature in this article such that they will be able to go on and explore the biology of this fascinating transcription factor in depth.

Overview of the NF-κB pathway

The five members of the mammalian NF-κB family, p65 (RelA), RelB, c-Rel, p50/p105 [NF-κB1], and p52/p100 [NF-κB2], exist in unstimulated cells as homo- or heterodimers bound to IκB family proteins. NF-κB proteins are characterized by the presence of a conserved 300-amino acid Rel homology domain (RHD) that is located toward the N terminus of the protein and is responsible for dimerization, interaction with IκBs, and binding to DNA [Fig. 1]. Binding to IκB prevents the NF-κB:IκB complex from translocating to the nucleus, thereby maintaining NF-κB in an inactive state. NF-κB signaling is generally considered to occur through either the classical or alternative pathway [for review, see Bonizzi and Karin 2004]. In the classical pathway of NF-κB activation, for example, upon stimulation by the proinflamma-

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The cytokine tumor necrosis factor (TNF), signaling pathways lead to activation of the IkB kinase (IKK) complex, which then phosphorylates IkB proteins on two N-terminal serine residues (Fig. 2A). In the alternative pathway, IKK is activated and phosphorylates p100 (Fig. 2B). Phosphorylated IkBs are recognized by the ubiquitin ligase machinery, leading to their polyubiquitination and subsequent degradation, or processing in the case of p100, by the proteasome (for review, see Karin and Ben-Neriah 2000). The freed NF-κB dimers translocate to the nucleus, where they bind to specific sequences in the promoter or enhancer regions of target genes. Activated NF-κB can then be down-regulated through multiple mechanisms including the well-characterized feedback pathway whereby newly synthesized IkB proteins bind to nuclear NF-κB and exports it out to the cytosol.

There are seven IkB family members—IκBα, IκBβ, BCL-3, IκBε, IκBγ, and the precursor proteins p100 and p105—which are characterized by the presence of five to seven ankyrin repeats that assemble into elongated cylinders that bind the dimerization domain of NF-κB dimers (Fig. 1; Hatada et al. 1992). The crystallographic structures of IkBα and IkBβ bound to p65/p50 or p65/c-Rel dimers revealed that the IkB proteins mask only the nuclear localization sequence (NLS) of p65, whereas the NLS of p50 remains accessible (Huxford et al. 1998; Jacobs and Harrison 1998; Malek et al. 2001, 2003). The presence of this accessible NLS on p50 coupled with nuclear export sequences (NES) that are present on IκBα and p65 results in constant shuttling of IκBα/NF-κB complexes between the nucleus and the cytoplasm, although the steady-state localization is in the cytosol (Johnson et al. 1999; Huang et al. 2000). The dynamic balance between cytosolic and nuclear localization is altered upon IκBα degradation, because it removes the contribution of the IκB NES and exposes the masked NLS of p65, resulting in predominantly nuclear localization of NF-κB.

Degradation of IkB is a tightly regulated event that is initiated upon specific phosphorylation by activated IKK. The IKK activity in cells can be purified as a 700–900-kDa complex, and has been shown to contain two kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit, NEMO [NF-κB essential modifier] or IKKγ (for reviews, see Rothwarf and Karin 1999; Ghosh and Karin 2002). In the classical NF-κB signaling pathway, IKKβ is both necessary and sufficient for phosphorylation of IκBα on Ser 32 and Ser 36, and IκBβ on Ser 19 and Ser 23. The role of IKKα in the classical pathway is unclear, although recent studies suggest it may regulate gene expression in the nucleus by modifying the phosphorylation status of histones. The alternative pathway, however, depends only on the IKKα subunit, which functions by phosphorylating p100 and causing its inducible processing to p52 (Fig. 2B). The alternative path-

**Figure 1.** Schematic representation of NF-κB, IkB, and IKK proteins family of proteins. Members of the NF-κB, IkB, and IKK proteins families are shown. The number of amino acids in each protein is indicated on the right. Presumed sites of cleavage for p100 (amino acid 447) and p105 (amino acid 433) are shown. Phosphorylation and ubiquitination sites on p100, p105, and IkB proteins are indicated. (RHD) Rel homology domain; (TAD) transactivation domain; (LZ) leucine zipper domain on IKKα/β and Rel-B; (GRR) glycine-rich region; (HLH) helix-loop–helix domain; (Z) zinc finger domain; (CC1/2) coiled-coil domains; (NBD) NEMO-binding domain; (α) α-helical domain.
way is activated in response to a subset of NF-κB inducers including LTβ and BAFF.

Upon phosphorylation by IKKs, IκB proteins are recognized and ubiquitinated by members of the Skp1–Cullin–Roc1/Rbx1/Hrt-1–F-box [SCF or SCRF] family of ubiquitin ligases [see Ben-Neriah 2002]. βTrCP [E3RS or Fbw1a], the receptor subunit of the SCF family ubiquitin ligase machinery, binds directly to the phosphorylated E3 recognition sequence [DS∗GXXS∗] on IκB [Yaron et al. 1997, 1998; Fuchs et al. 1999; Hatakeyama et al. 1999; Kroll et al. 1999; Suzuki et al. 1999; Winston et al. 1999; Wu and Ghosh 1999]. Recognition of IκBα leads to polyubiquitination at conserved residues, Lys 21 and Lys 22 on IκBα, by the E3 SCFβ-TrCP and the E2 UbcH5 [Alkalay et al. 1995; Scherer et al. 1995; DiDonato et al. 1996]. Although it is commonly believed that degradation of IκBα is a cytoplasmic event, βTrCP1 is almost exclusively nuclear with its receptor site occupied by hnRNP-U [Davis et al. 2002]. This finding has led to the suggestion that phosphorylated IκBα must out-compete hnRNP-U for binding to βTrCP1. However, βTrCP2, a highly homologous isoform of βTrCP1, is localized in the cytoplasm and can bind IκBα, although it has lower ligase efficiency than βTrCP1 [Suzuki et al. 2000; Davis et al. 2002]. Cells from βTrCP1-deficient mice display partially decreased rates of IκBα and IκBβ degradation, indicating that this βTrCP1 function can be partially compensated for by βTrCP2 [Nakayama et al. 2003]. Determination of the actual role of β-TrCP isoforms in IκB degradation will have to await the generation of mice lacking both isoforms.

The precursor protein p105 undergoes constitutive processing, as opposed to degradation, via the proteasome, through a cotranslational mechanism [Fan and Maniatis 1991; Palombella et al. 1994; L. Lin et al. 1998]. Limited proteolysis of the precursor protein, which generates p50, is dependent on the presence of a glycine-rich region [GRR] between amino acids 376 and 404 that serves as a stop signal for proteolysis [Lin and Ghosh 1996; Orian et al. 1999]. Whether p105 can also undergo inducible processing remains contentious. Multiple reports have demonstrated IKKβ-dependent, and IKKα-independent, phosphorylation of p105 C-terminal serines including Ser 923 and Ser 927, followed by inducible degradation, although there is no definitive evidence that this degradation of p105 has a functional role [Fujimoto et al. 1995; MacKichan et al. 1996; Heissmeyer et al. 1996].
1999, 2001; Orian et al. 2000; Salmeron et al. 2001; Lang et al. 2003; Cohen et al. 2004). It has been suggested that similar to IkB, phosphorylation of p105 leads to SCF^B-TrCP binding, resulting in polyubiquitination and degradation of the protein. Polyubiquitination of p105 leading to degradation involves multiple lysine residues, and is dependent on an acidic region between residues 445 and 453 (Harhaj et al. 1996; Orian et al. 1999; Cohen et al. 2004). In contrast, partial processing leading to p50 may be carried out by a different ubiquitin ligase machinery [Amir et al. 2002; Lang et al. 2003; Cohen et al. 2004]. It was recently demonstrated that SCF^B-TrCP is not responsible for signal-induced processing, and it has been suggested that this event is independent of ubiquitination [Cohen et al. 2004]. The p105 precursor protein forms multiple heterodimeric and homodimeric complexes [Ghosh 2004], and this association with NF-кB proteins inhibits constitutive processing [Harhaj et al. 1996; Cohen et al. 2001]. Most likely, processing of p105 is partially determined by the milieu of NF-кB dimers present in any given tissue.

Processing of p100 bears some similarity to both IkB and degradation and p105 processing. The phosphorylation of p100 has, so far, only been shown to be catalyzed by IKKs acting downstream from NF-кB inducing kinase [NIK; Fig. 2B; Sentftleben et al. 2001a]. Unlike p105, p100 processing is a tightly regulated event, with only minimal constitutive processing in unstimulated cells [Heusch et al. 1999]. Interestingly, it appears that NIK can act both as an IKK-activating kinase as well as a docking protein linking IKKs to p100 [Xiao et al. 2004]. The C-terminal death domain [DD] of p100 has been shown to function as a processing inhibitory domain [PID], and expression of a p100 lacking this domain results in increased constitutive processing in a manner dependent on nuclear shuttling [Xiao et al. 2001; Liao and Sun 2003]. Similar to IkB, phosphorylation of p100 leads to the recruitment of SCF^B-TrCP^B, polyubiquitination of Lys 855 in a region with sequence homology to Lys 22 of IkB, and subsequent processing to p52 [Fong and Sun 2002; Amir et al. 2004; Cohen et al. 2004]. Like p105, the p100 GRR is also required for partial processing yielding p52 and release of active p52:NF-кB complexes [Heusch et al. 1999].

Following degradation of IkB, the released NF-кB is able to bind promoter and enhancer regions containing кB sites with the consensus sequence GGGRNNYYCC [N = any base, R = purine, and Y = pyrimidine]. The crystal structures of NF-кB dimers bound to the кB enhancer reveal how both immunoglobulin-like domains that make up the RHD contact the DNA of the кB site. The N-terminal Ig-like domain is primarily responsible for sequence specificity of NF-кB, whereas hydrophobic residues within the C-terminal domain form the dimerization interface [Ghosh et al. 1995; Muller et al. 1995; F.E. Chen et al. 1998; Y.Q. Chen et al. 1998; Huxford et al. 1998; Huang et al. 2001]. RelB, c-Rel, and p65 contain a transactivation domain [TAD] located toward the C terminus that is necessary for transactivation by these proteins. Homodimers of p52 and p50 lack TADs and hence have no intrinsic ability to drive transcription. In fact, binding of p52 or p50 homodimers to кB sites of resting cells leads to repression of gene expression [Zhong et al. 2002]. The repressive function of p50 or p52 homodimers may provide a threshold for NF-кB transactivation that can be regulated through the expression and processing of the p100 and p105 precursors. The TADs on p65, c-Rel, and RelB promote transcription by facilitating the recruitment of coactivators and the displacement of repressors. The function of TADs is enhanced through direct modifications of NF-кB including phosphorylation, and represents another layer of regulation of the NF-кB-mediated transcriptional response [for review, see Chen and Greene 2004].

**Biological roles of NF-кB and IkB proteins**

All of the mammalian Rel and IkB family members have been knocked out in mice, and many conditional knockouts and multigene knockouts have also been generated. The phenotype of these various mice with regard to their immune system function and roles in the regulation of apoptosis has recently been reviewed in detail [Kucharczak et al. 2003; Bonizzi and Karin 2004]. Therefore, we provide here a succinct overview of the function of each pathway component based on the relevant genetic experiments in support of such a role.

Genetic studies first revealed the crucial role of p65 in mediating protection from apoptosis during TNF signaling. p65^−/− mice exhibit lethality caused by liver degeneration at gestational day 15–16 [Beg et al. 1995b]. The insult leading to the observed liver apoptosis was shown to be TNF signaling in the developing liver, as crossing p65^−/− mice with either TNFR1^−/− [Doi et al. 1999] or TNFR2^−/− [Alcamo et al. 2001] mice rescues the liver phenotype and allows the study of the effects of p65 knockout on other tissues. p65/TNFR1 double knockouts have increased susceptibility to bacterial infection, highlighting the role of p65 in innate immune responses and the initiation of innate immune responses by non-hematopoietic cells [Alcamo et al. 2001]. The knockouts of p65 were also used to show its importance in class switching in B cells and lymphocyte proliferation following various stimuli in chimeric mouse models [Doi et al. 1997].

In contrast to p65-deficient mice, p50 (NF-кB1)-deficient mice do not show any developmental defects [Sha et al. 1995]. Instead, p50^−/− mice exhibit decreased immunoglobulin production and defective humoral immune responses. B cells from these mice do not respond efficiently to LPS, underscoring the role of the classical p65/p50 heterodimer in Toll/IL-1 signaling pathways, whereas p50 is redundant for antiapoptotic pathways.

Mice lacking p52 (NF-кB2) fail to develop normal B-cell follicles and germinal centers and have additional defects in their splenic architecture and Peyer’s patch development [Caamano et al. 1998; Franzoso et al. 1998; Paxian et al. 2002]. These mice display normal B-cell maturation and undergo class-switch recombination, but generate inadequate humoral responses to various
T-cell-dependent antigens. In addition, p52 knockouts fail to generate appropriate class-switched antibodies following influenza infection. Rescue of the T-cell-dependent response by adoptively transferring p52-deficient cells into rag-1^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^
induced in response to NF-κB activation (Simeoni-dis et al. 1997; Whiteside et al. 1997), it appears that it may function in regulating later phases of NF-κB gene activation by p65:Rel complexes. IκBα is actually the C-terminal region of mouse p105 (Inoue et al. 1992; Gerondakis et al. 1993; Grumont and Gerondakis 1994). It is still unclear whether IκBα, which is synthesized as an alternate mRNA using a separate promoter, has a defined biological role. Finally, IκBβ (also called MAIL/INAP), perhaps the eighth member of the mammalian IκB family, is found in the nucleus and is expressed in response to IL-1 and TLR stimulation of NF-κB but not TNF (Kitamura et al. 2000; Haruta et al. 2001; Yamazaki et al. 2001; Eto et al. 2003; Muta et al. 2003). Although IκBβ has weak homology to BCL-3 and the other IκBs, there is not yet any direct evidence that it is functionally an IκB family member, as it has not yet been shown to interact with any Rel family members.

Signaling pathways to NF-κB

Numerous pathways lead to the activation of NF-κB. Almost universally, these pathways proceed via activation of IKK, degradation of IκB, and enhancement of the transcriptional activity of NF-κB. However, there is significant variability in fundamental aspects of these pathways, for example, TNFα signaling via TNFR1 results in the rapid activation of IKK and nearly complete degradation of IκBα within ~10 min, whereas signal-induced degradation of IκBα through the TCR takes nearly 45 min. Furthermore, individual NF-κB responses can be characterized as consisting of waves of activation and inactivation of the various NF-κB family members (Hoffmann et al. 2002, 2003). This is because of sustained activation of the IKK complex, and its selectivity for different IκBs, as well as the differential regulation of IκB expression by NF-κB dimers. The baseline complement of NF-κB complexes present in a cell can therefore influence the nature of the transcriptional response to a given stimulus. The transcriptional activity and specificity of NF-κB are also differentially regulated. Summation of these effects results in unique transcriptional responses to a given stimulus in a particular cell type. A careful examination of the most-studied pathways—TNFR, TLR/IL-1R, TCR, and BCR—highlights some of these differences, but also reinforces the commonalities that characterize signaling to NF-κB in general (Fig. 3).

Figure 3. Major signaling pathways that lead to NF-κB activation. Signal transduction pathways emanating from TNF receptor (A), Toll/IL-1 receptor (B), the BCR (C), and intermediary proteins involved. [Lower left] An outline of pathways leading to NF-κB as a consequence of cell stress and DNA damage is also indicated. The activation of the TCR is shown using cross-linking anti-CD3/CD28 antibodies.
TNFα: a model NF-κB stimulus

The TNFR superfamily consists of at least 19 ligands and 29 receptors and exhibits a remarkable diversity in tissue distribution and physiology [for review, see Aggarwal 2003]. These receptor/ligand pairs initiate a variety of biological responses, primarily through activation of inducible transcription factors such as NF-κB and AP-1 [for review, see Wajant et al. 2003]. TNFα is probably the most widely studied member of this family of cytokines and performs multiple roles in innate and adaptive immune responses. Most notably, through activation of NF-κB, TNF signaling directly regulates the expression of anti-apoptotic genes such as cIAP1/2 and Bcl-XL [for review, see Kucharczak et al. 2003]. If NF-κB signaling is blocked, then exposure to TNFα induces rapid apoptosis in most cell types. Aberrant TNF signaling has been implicated in many disease states, and anti-TNF antibodies are in clinical use for the treatment of inflammatory conditions such as rheumatoid arthritis [for review, see Aggarwal 2003]. Therefore, a detailed understanding of IKK activation through this pathway may provide an opportunity to develop targeted inhibitors with the potential to treat such conditions. However, despite the tremendous clinical relevance of this pathway, and enormous effort in numerous laboratories, there are still significant gaps in our understanding of the mechanisms that underlie TNFα-mediated activation of NF-κB.

TNF family receptors lack intrinsic enzymatic activity. Instead, signaling is achieved by recruitment of intracellular adapter molecules that associate with the cytoplasmic tail of the TNFR in a signal-dependent manner [for review, see Aggarwal 2003; Wajant et al. 2003]. The recruitment of TNFR1 to membrane microdomains, referred to as lipid rafts, with subsequent assembly of the signaling complex, is necessary for signaling to NF-κB and prevention of apoptosis [Hueber 2003; Legler et al. 2003]. Ligation of TNFR1 by trimeric TNFα causes aggregation of the receptor and dissociation of Silencer of death domain (SODD), an endogenous inhibitor of TNF receptor activity, allowing binding of the TNFR-associated death domain protein (TRADD, Fig. 3A; Jiang et al. 1999). SODD may, however, play a redundant role, as studies on SO-DD−/− mice reveal marginal effects on TNFα signaling to NF-κB [Endres et al. 2003; Takada et al. 2003]. TRADD subsequently recruits downstream adapters that result in IKK, p38, JNK, and caspase activation. One critical set of adapter molecules that are recruited to TRADD through direct interaction is the TNF-receptor-associated factor (TRAF) family [for review, see Kelliher et al. 1998; Pober and Bradley 2001; Chung et al. 2002; Dempsey et al. 2003].

Multiple members of the TRAF family including TRAF2, TRAF3, and TRAF5 have been implicated in TNF signaling, however elucidating their functional roles has been complicated because of diverse homotypic and heterotypic interactions that can occur between members of this family. TRAF2 is inducibly recruited to the TNFR and interacts efficiently with TRADD [Hsu et al. 1996]. Surprisingly, TRAF2-deficient mice have intact TNF signaling to NF-κB; instead, TRAF2 appears to mediate signaling to AP-1 [Yeh et al. 1997]. TRAF5 knockouts also exhibit normal NF-κB activation by TNF; however, TRAF2/5 double knockout cells have substantially reduced TNF-induced IKK activation [Yeh et al. 1997; Nakano et al. 1999; Tada et al. 2001]. Therefore, TRAF2 and TRAF5 appear to play a redundant role in TNF signaling to NF-κB, although the nature of this role remains to be determined. It has been suggested that TRAF2 recruits the IKK complex following TNFα treatment through a direct interaction with the leucine zipper of IKKα or IKKβ [Devlin et al. 2001]. TRAF2 also interacts with the serine/threonine kinase, Receptor interacting protein 1 (RIP1), which can also be independently recruited to TRADD. RIP1 is essential for TNF-induced NF-κB activation [Hsu et al. 1996], and RIP-deficient cells exhibit increased apoptosis following TNFα stimulation owing to their inability to activate NF-κB [Kelliher et al. 1998]. Intriguingly, RIP may function as a scaffold molecule in TNF signaling because its kinase activity is dispensable for NF-κB activation [Hsu et al. 1996; Ting et al. 1996; Devlin et al. 2000]. RIP1 can bind directly to NEMO and thereby recruit IKK to the TNFR1 signaling complex independent of TRAF2 [Zhang et al. 2000]. Interestingly, IKK recruitment in the absence of RIP, presumably through TRAF2, is not sufficient for IKK activation [Devlin et al. 2001], demonstrating that RIP has a role in addition to or independent of simple recruitment of the IKK complex. RIP may nucleate the assembly of a signaling complex that induces IKK activation through oligomerization of NEMO and subsequent autophosphorylation of IKK [Delhase and Karin 1999]. Alternatively, the assembly of a signaling complex may facilitate NF-κB signaling by bringing the IKK complex into close proximity with an IKK kinase.

An intriguing feature of signaling by TNF is its ability to stimulate both death and survival. As noted above, the balanced induction of both pathways usually leads to activation of cells; however, if activation of NF-κB, and consequently the survival pathways, are blocked, TNF becomes a potent apoptosis-inducing factor. In general, direct up-regulation by NF-κB of factors such as IAPs is responsible for the antiapoptotic effect of NF-κB [for review, see Kucharczak et al. 2003]. It appears, however, that the two main target transcription factors of TNF signaling, NF-κB and AP-1, also participate directly in arbitrating the choice between survival and death. It has been observed that prolonged activation of JNK/AP-1 leads to apoptosis [Guo et al. 1998; De Smaele et al. 2001; Tang et al. 2001]. NF-κB induction can lead to the synthesis of certain effectors that inhibit the JNK pathway, thereby limiting JNK/AP-1 activation. One such NF-κB inhibiting factor is GADD45β, which inhibits JNK signaling by blocking the upstream kinase MKK7 [De Smaele et al. 2001; Jin et al. 2002; Papa et al. 2004]. Although a more recent study showed that activation of JNK is similar to wild-type cells in GADD45β−/− MEFs [Jin et al. 2002], it is possible that GADD45β may cooperate with some other NF-κB-induced inhibitor of JNK. For example, X-chromosome-linked inhibitor of apoptosis...
[XIAP] is induced by NF-κB, and, in addition to inhibiting multiple proapoptotic caspases, it also blocks NF-κB activation [Tang et al. 2001]. Another NF-κB-regulated gene, cFLIP (Casper), inhibits JNK at the level of TRAF/ RIP [Shu et al. 1997; Kreuz et al. 2001]. Furthermore, the roles of JNK and NF-κB are not entirely antagonistic, as expression of the antiapoptotic protein cIAP1 is coregulated by JNK and NF-κB, and consequently TNFα-induced apoptosis is increased in JNK double-knockout cells [Lamb et al. 2003; Ventura et al. 2003]. Interestingly, TRAF-2-deficient cells are more sensitive to TNFα-induced cell death although NF-κB signaling remains intact. This may reflect the requirement for TRAF-2 binding to cIAP in order for it to be localized to its site of function or the requirement of JNK-mediated JunD activation for appropriate expression of cIAP [Shu et al. 1996; Wang et al. 1998; Lamb et al. 2003].

Lymphotoxin-βR, BAFF-R, and CD40; TNFR family members that signal to NF-κB through the alternative pathway

The recent discovery of the alternative signaling pathway leading to inducible p100 processing has provided a novel addition to the well-studied pathway of NF-κB activation through the degradation of IκB proteins [for review, see Bonizzi and Karin 2004]. This represents an important step forward in understanding how NF-κB family members might be differentially regulated. The alternative pathway is unique in that it is independent of IKKβ and NEMO [Claudio et al. 2002; Dejardin et al. 2002]. Instead, the functional IKK is thought to be IKKa homodimers, which selectively phosphorylate p100 associated with RelB. Therefore, processing of p100 releases a subset of transcriptionally active NF-κB dimers, consisting mainly of p52:RelB [Dejardin et al. 2002; Xiao et al. 2004]. In contrast to the other pathways discussed here, in the alternative pathway the mechanism of IKKa activation is known. NF-κB inducing kinase (NIK) is responsible for directly phosphorylating and activating IKKa [Senftleben et al. 2001a; Xiao et al. 2001]. However, events that occur upstream of NIK are unclear. The key question concerning signaling by these stimuli is how they are channeled to NIK and IKKa, even though their receptor signaling domains resemble those of other TNF family members. Because LTβ, BAFF, and CD40L also activate the classical pathway, it would appear that the intracellular signaling domains of these receptors possess additional sequence motifs that allow their coupling to NIK and the alternative pathway. The creation and systematic analysis of chimeras between these receptors and those that only signal through the classical pathway will probably be necessary to elucidate the mechanism by which the alternative pathway is activated.

Toll/IL-1 receptor signaling to NF-κB

Signaling to NF-κB mediates multiple aspects of innate and adaptive immunity [for review, see Ghosh et al. 1998; Silverman and Maniatis 2001; Bonizzi and Karin 2004]. NF-κB plays an essential role in early events of innate immune responses through Toll-like receptor (TLR) signaling pathways. TLRs are evolutionarily conserved Pattern recognition receptors (PRRs) that recognize conserved Pathogen-associated microbial patterns (PAMPs) present on various microbes [for review, see Janeway and Medzhitov 2002; Kopp and Medzhitov 2003; Takeda et al. 2003]. The role of TLRs as arbitrators of the self–non-self decision means that they play a central role in innate immunity as well as in the initiation of the adaptive immune responses. To date, 11 mammalian TLRs have been described, and each of these signals to NF-κB. These receptors have varied tissue distribution and recognize many different PAMPs including LPS, dsRNA, nonmethylated CpG DNA, and flagellin. Some members of the TLR family are also capable of heterodimerization, thereby further expanding the repertoire of molecules that are recognized. Significant progress has been made over the past couple of years in deciphering the relevant signaling pathways that operate downstream of TLRs [for review, see Barton and Medzhitov 2003]. The intracellular domain of Toll-like receptors bears strong homology with the intracellular domain of the IL-1 receptor, and it is this shared Toll-IL-1R (TIR) domain that mediates interaction with downstream signaling adapters that lead to activation of three key transcription factors, NF-κB, AP-1, and IRF5.

TLR signaling is initiated by the recruitment of cytosolic adapters that all share the TIR domain [for review, see Dunne and O’Neill 2003]. MyD88 was the first TIR domain containing adapter protein characterized and was shown to interact with the TIR domain on TLR/IL-1R cytoplasmic tails by homotypic interaction. MyD88 is crucial for normal NF-κB induction in response to IL-1, IL-18, and LPS [TLR4, Adachi et al. 1998; Kawai et al. 1999]. MyD88 recruitment to TLR4 following receptor aggregation leads to recruitment of another TIR-domain-containing adapter, TIRAP or Mal [Fig. 3B]. TIRAP mediates NF-κB activation downstream of TLR2 and TLR4, but not IL-1R or other TLRs [Horn et al. 2002; Yamamoto et al. 2002]. Currently it is not known how TIRAP selectively acts only in a subset of MyD88-mediated signaling pathways [for review, see McGwire and O’Neill 2004]. MyD88 also contains an N-terminal death domain (DD) that recruits downstream DD-containing signaling molecules such as the serine/threonine kinase IRAK [for review, see Janssens and Beyaert 2003]. The exact role played by IRAK family members is somewhat enigmatic because like RIP, IRAK-1 kinase function is also dispensable for its role in TLR/IL-1 signaling [Knop and Martin 1999; X. Li et al. 1999]. IRAK-4 is also required for signaling from TLR and IL-1, likely upstream of IRAK-1 (Suzuki et al. 2002). IRAK recruitment and activation are necessary to bring TRAF6 into the signaling complex, although exactly how this is achieved remains mysterious [Qian et al. 2001; Takaesu et al. 2001]. TRAF6 is recruited to TLR/IL-1R and is required for MyD88-dependent activation of NF-κB [Cao et al. 1996; Wescie et al. 1997; Wu and Arron 2003]. TRAF6-deficient cells exhibit a complete loss of NF-κB DNA binding induced.
The link between TRAF6 and the IKK complex has remained controversial. Two sets of adapters have been proposed to link TRAF6 with IKK. The first involves the Transforming growth factor β activated kinase 1 (TAK1), and two associated adapter proteins TAB1 and TAB2 (Takaesu et al. 2000). The major support for this signaling module came from studies that indicated that they could be copurified with TRAF6 in cells (Wang et al. 2001). The presence of a RING finger domain in TRAF6 led to the suggestion that TRAF6 could function as a ubiquitin ligase (Deng et al. 2000). Upon stimulation, TRAF6 would ubiquitinate itself or components of the TAK1/TAB1/TAB2 complex, thereby priming them for IKK activation (Sun et al. 2004). However, as of yet there is no genetic evidence in mammalian systems supporting such a critical role for TAK1 in TLR/IL-1 signaling. Intriguingly, a recent study indicated that RNAi-mediated knock-down of TAK1 inhibited signaling from both IL-1 and TNF receptors, which was surprising because TRAF6 is not involved in the TNF pathway (Takaesu et al. 2003). One possibility is that TAK1 function is not limited to TRAF6, but rather may extend to other TRAFs that also possess RING finger domains. A more recent report has implicated TAK1 as an essential component in TCR signaling, raising the possibility that contrary to current belief, TRAF proteins are also involved in antigen-receptor signaling (Sun et al. 2004). However, the phenotypes of TAB1 and TAB2 knockouts fail to support any role for these proteins in TLR/IL-1 and TNF signaling. Knockout of TAB1 and TAB2 both lead to embryonic lethality at embryonic days 17 (E17) and 12.5 (E12.5), respectively (Komatsu et al. 2002; Sanjo et al. 2003). Fibroblasts isolated from the embryos display completely normal signaling in response to TNF and IL-1 (Sanjo et al. 2003; M.S. Hayden and S. Ghosh, unpubl.). Knocking out TAK1 causes embryonic lethality at E10, thus preventing signaling studies. Therefore, although it remains possible that future development and characterization of conditionally knocked out TAK1 cells will support the proposed role in TLR/IL-1, TNF, and TCR signaling, it is clear that there are significant complexities yet to be resolved.

Besides the TAK1/TAB1/TAB2 proteins, another protein that has been reported to act as a bridge between TRAF6 and the IKK complex is ECSIT [evolutionarily conserved signaling intermediate in toll pathways]. ECSIT was identified in a yeast two-hybrid screen as a binding partner of TRAF6 and was shown to be required for TLR and IL-1 signaling, but not TNF-signaling, using RNAi knock-down techniques (Kopp et al. 1999; Xiao et al. 2003). Knockout of ECSIT leads to very early embryonic lethality, thereby preventing isolation of cells suitable for signaling studies. However, the knockout of ECSIT displays phenotypes that are identical to those seen in knockouts of Bmp receptor-1, a member of the TGFβ-receptor family that is known to function in early development. In fact, subsequent analysis showed that ECSIT can function as a coactivator for effectors of Bmp/TGFβ signaling, namely, the Smad transcription factors (Xiao et al. 2003). This surprising finding raises the possibility that ECSIT may regulate both TLR and TGF/Bmp pathways, and hence may help provide an explanation for why these pathways cross-repress one another (Moustakas and Heldin 2003). It is also important to point out that the TAK/TAB proteins were also initially identified and characterized as intermediates in TGFβ signaling, and hence it is possible that this link between adapters in TLR and TGF signaling pathways may be more extensive than currently imagined.

The initial studies with ECSIT suggested that it might function in TLR signaling by recruiting and activating the kinase MEKK1, which is also involved in TGFβ signaling (Kopp et al. 1999; Zhang et al. 2003). However, knockouts of MEKK1 do not display any overt phenotype in TLR or TNF signaling, suggesting that some other kinase might be involved (Xia et al. 2000; Yujiri et al. 2000). More recently, MEKK3 has been strongly implicated in TLR signaling. MEKK3-deficient cells do not transcribe IL-6 following TLR4 or IL-1R stimulation and exhibit delayed and weak NF-κB DNA binding following LPS stimulation (Huang et al. 2004). Although TRAF6 and MEKK3 inducibly associate in TLR4 signaling, the mechanisms that regulate this process are not known (Huang et al. 2004). Therefore, it is possible, although not proven, that ECSIT exerts its role in TLR signaling by somehow modulating the function of MEKK3.

Multiple TLRs are also capable of signaling in the absence of MyD88. LPS stimulation in MyD88−/− cells results in NF-κB activation with slower kinetics than from normal TLR signaling and leads to expression of only a subset of target genes (Kawai et al. 1999). TRIF (TICAM-1), a TIR-domain-containing adapter, mediates activation of NF-κB in the absence of MyD88 when cells are stimulated through TLR3 and TLR4 (Oshiumi et al. 2003). TRIF expression is regulated by NF-κB and is therefore induced by TLR and IL-1R signaling (Hardy et al. 2004). Studies using cells from TRIF-deficient mice demonstrate that TRIF is required for early and late NF-κB responses and IRF3 responses to LPS, but not for JNK activation (M. Yamamoto et al. 2003a; Akira 2004). Reconstitution of TRIF−/− cells with a mutant form lacking the TRAF-binding domain restores induction of IFNβ-responsive genes, via activation of IRF3, but not NF-κB activation, indicating that TRIF is the point of divergence in signaling to NF-κB and IRF3 by TLR4. Recently it has been reported that TRIF binds RIP1 and that RIP1/−/− MEFs have decreased NF-κB signaling from TLR3-poly[I:C] (Meylan et al. 2004). Finally, another TIR-domain-containing adapter, TRAM (TRIF-related adapter molecule), functions upstream of TRIF in MyD88-independent signaling from TLR4. TRAM is required for IRF-3 activation and for the delayed phase of NF-κB activation following TLR4 engagement. TLR4-induced IRAK activation by MyD88, however, is unaffected by the absence of TRAM (M. Yamamoto et al. 2003b). TRAM does not function in TLR3 or IL-1R signaling pathways (Fitzgerald et al. 2003; M. Yamamoto et al. 2003b).
Two divergent members of the IKK family, IKKi (IKKe) and TBK1 (T2K), have been implicated downstream of TRIF in signaling to IRF-3 and IRF-7, following engagement of TLR3 and TLR4 [Hemmi et al. 2004; Sharma et al. 2003]. Although initially both of these kinases were implicated in regulation of NF-κB activity, recent results have cast doubt on those conclusions. Studies using knockout cells indicate that neither is required for NF-κB activation by LPS or TNFα [Akira 2004; Hemmi et al. 2004]. Also in contrast to the study reporting the knock-out of TBK1, a recent study reported that there was no discernible effect on the transcriptional activity of NF-κB in TBK1 knockout cells following stimulation with multiple PAMPs [McWhirter et al. 2004]. The reason for this discrepancy remains to be resolved. IKKi−/− cells have normal induction of IRF3 following stimulation with LPS [hence TLR4], whereas TBK1−/− cells do not [Hemmi et al. 2004]. IKKi expression is regulated by NF-κB, and it appears that IKKi may be constitutively active once expressed [Mercurio 2004]. IKKi may facilitate CCAAA/enhancer-binding protein δ (C/EBPδ) pathways that contribute to the expression of a subset of genes that are induced by IL-1, LPS, TNF, or PMA [Kravchenko et al. 2003]. Therefore, TBK1 but not IKKi mediates TLR signaling to IRF-3, likely through direct phosphorylation of IRF-3, and neither kinase appears to be directly involved in TLR signaling to NF-κB.

The Toll-signaling pathway can also be negatively regulated by proteins that are induced or activated upon TLR signaling and therefore may help to limit signaling from these receptors. For example, Tollip is an adapter protein found in association with IRAK in the IL-1R signaling pathway [Burns et al. 2000; Zhang and Ghosh 2002]. Tollip binds to IRAK, and to TLR2, TLR4, or IL-1R upon signaling. Activation of IRAK by MyD88 results in autophosphorylation of IRAK and phosphorylation of Tollip, causing their dissociation [Bulut et al. 2001; Zhang and Ghosh 2002]. Tollip can suppress TLR/IL-1R signaling when overexpressed and, therefore, functions as a negative regulator of IRAK activity. It is not known whether Tollip functions in down-regulation of TLR signaling or acts as a constitutive inhibitor of IRAK. It has been suggested that high levels of Tollip in the intestinal epithelium help to prevent inflammation in response to commensals [Melmed et al. 2003]. Another well-characterized negative regulator is a member of the IRAK family, IRAK-M. IRAK-M is inducibly expressed in myeloid cells and was initially shown to induce NF-κB signaling when overexpressed [Wesche et al. 1999]. However, in knockout mice, TLR and IL-1R signaling to NF-κB is enhanced, and the cells do not become tolerant of repeated LPS stimulation [Kobayashi et al. 2002]. IRAK-M prevents the release of IRAK-1 and IRAK-4 from the TLR/MyD88 signaling complex, and therefore inhibits the association between IRAKs and TRAF6. Further upstream, alternative splice variants of MyD88 have been reported to inhibit TLR signaling by competitively binding IRAK4 [Janssens et al. 2002, 2003; Burns et al. 2003]. Finally, at the level of the membrane, SIGIRR (TIR8), a member of the IL-1R family that contains an extracellular Ig domain, has been shown to decrease TLR/IL-1R signaling [Thomassen et al. 1999; Wald et al. 2003]. In SIGIRR−/− cells, treatment with either LPS or IL-1 leads to enhanced and prolonged activation of both NF-κB and JNK. SIGIRR binds to other Toll/IL-1 receptors and interacts with IRAK and TRAF6 [Wald et al. 2003]. It remains to be seen whether it inhibits signaling by competing for adapters or preventing the dissociation of IRAK from the receptor in a manner analogous to IKK-M. Interestingly, SIGIRR is most abundantly expressed in epithelial cells, leading one to speculate that it may suppress TLR signaling at sites of exposure to commensal bacteria.

**BCR and TCR signaling to NF-κB**

Signaling from BCRs and TCRs is critical for mounting adaptive immune responses and has consequently been of great interest to numerous laboratories. Activation of NF-κB downstream of BCR and TCR allows antigen-specific proliferation and maturation of lymphocytes into effector cells. Signaling through these two antigen receptors is functionally analogous, although the molecular details differ. From a clinical standpoint, understanding how these signaling pathways operate may provide novel targets for therapies directed at autoimmunity, inflammatory disorders, and transplant rejection.

Signaling through the TCR, or analogously through the BCR, and various costimulatory molecules leads to NF-κB activation [for review, see Ruland and Mak 2003]. TCR ligation induces phosphorylation of key residues on ITAMs present on TCR or CD3 (Fig. 3C). Phosphorylated ITAMs recruit SH2-domain-containing adapters, most notably the Syk family tyrosine kinases Lck and Zap70. However, the link between the receptor proximal tyrosine kinases and NF-κB is poorly defined. Recent studies have implicated several potential signaling intermediates that appear to comprise a novel signaling pathway, including PKCθ, CARMA1/CARD11, BCL10, and MAL1 [for reviews, see Lucas et al. 2004; Simeoni et al. 2004; Thome 2004]. The IKK complex is rapidly recruited to the immunological synapse through an unknown mechanism and can be colocalized to the TCR by confocal immunofluorescence analysis [Khoshnan et al. 2000]. In Zap-70-deficient cells, NF-κB activation induced by TCR stimulation can be rescued with a NEMO-(SH2)2 chimera that targets NEMO to the immunological synapse, indicating that events downstream from Zap-70 are only required for IKK recruitment [Weil et al. 2003]. One intriguing hypothesis that has been proposed is that recruitment of the IKK complex is dependent on changes in cytoskeletal structure during formation of the immunological synapse [Weil et al. 2003]. Although the importance of scaffolding molecules in the MAPK pathway has been established, little is known thus far about how these processes affect NF-κB signaling downstream of antigen receptors.

It is clear from knockout studies that PKCθ is essential for activation of NF-κB by TCR [Sun et al. 2000], but the specific role played by PKCθ in T cells, and PKCβ1/2 in...
B cells (Guo et al. 2004), remains to be elucidated. PKCδ is specifically recruited to the immunological synapse, although how this specificity for PKCδ is achieved remains unclear because T cells express most of the other PKC isoforms. The exact role of PKCδ in the immunological synapse is also unclear, as is the mechanism responsible for recruiting PKCδ. PKCδ is capable of directly interacting with the IKK complex in primary T cells (Khoshnan et al. 2000), and it is possible that PKCδ might act as a scaffold/adapter to link events in the synapse with the other essential components in this pathway, namely, CARMA1/CARD11, BCL10, and MALT1. Knockouts of each of these genes leads to a specific block in NF-κB activation in response to antigen-receptor signaling, and therefore current efforts are focused on determining how these proteins are linked to the IKK complex (Ruland et al. 2001; Hara et al. 2003; Ruefli-Brasse et al. 2003).

The MAGUK family protein CARD11/CARMA-1 is required for PKCδ-mediated activation of NF-κB following TCR ligation (Gaide et al. 2002; Hara et al. 2003). BCL10, which interacts with MALT1 and cIAPs, is also critical for NF-κB activation via the BCR (Ruland et al. 2001). BCL10 interacts with CARMA1 and undergoes CARMA1-dependent phosphorylation, although CARMA-1 lacks kinase activity (Bertin et al. 2001; Gaide et al. 2001). BCL10 knockout mice are unable to activate NF-κB in response to antigen-receptor stimulation (Ruland et al. 2001). Interestingly, genetic evidence of a role for RIP2 has recently been reported in T-cell signaling (Ruefli-Brasse et al. 2004). RIP2 associates with BCL10 and is necessary for TCR-induced BCL10 phosphorylation and IKK activation. It is not yet clear how upstream mediators of TCR signaling may regulate RIP2 and, in turn, how RIP2 might affect IKK activity. BCL10 oligomerization has been implicated in IKK activation through a process that involves ubiquitination of NEMO (Zhou et al. 2004). This ubiquitination event appears to be mediated by MALT1 and possibly CARMA-1. A surprising recent finding is that TCR-induced ubiquitination of NEMO may depend on TRAF6, which has not previously been thought to play any role in TCR signaling, as T-cell deficits have not been reported in TRAF6-deficient mice (Lomaga et al. 1999; Sun et al. 2004). It is interesting to note that RIP2 associates with TRAF6, and one could speculate that this interaction has a functional role in IKK activation downstream of antigen receptor signaling (McCarthy et al. 1998).

**Cell stress**

Various intracellular stressors, both physiological and pathological, induce the activation of NF-κB [Fig. 3]. Understanding the pathways responsible will require a significant shift in thinking from traditional signaling paradigms, as there is not an initiating receptor ligation event. Although less conventional, the physiological relevance of these pathways is undeniable. A properly orchestrated response to genotoxic stress is a fundamental defense against transformation and cancer. However, the biological function of NF-κB in this response is not immediately clear. Although it is generally hypothesized that activation of NF-κB provides an antiapoptotic signal, thereby providing a window of opportunity for the cell to repair DNA damage, this has not been formally proven. In fact, under some circumstances, NF-κB signaling can play a proapoptotic role in response to stimuli such as UV irradiation (Campbell et al. 2004). Nevertheless, cell irradiation and DNA damage have long been known to activate NF-κB (Brach et al. 1991). However, considerable discrepancies exist in the experimental systems used to investigate cell stress, and this has hindered progress in this area. Many of the agents used to induce genotoxic stress do so through diverse perturbations of cell physiology and could potentially activate NF-κB through multiple mechanisms. Even when selectively induced, DNA damage elicits a highly complex cellular response that has been the subject of considerable research. The product of the gene mutated in ataxia-telangiectasia (ATM), a phosphoinositide 3-kinase-related kinase (PIKK), has a central role in sensing DNA doublestrand breaks [DSBs] and triggering the subsequent activation of the DNA repair machinery (for review, see Yang et al. 2004). It has been shown using ATM knockout cells, and cells from patients with ataxia-telangiectasia, that ATM is required for NF-κB activation following ionizing radiation (Li et al. 2001). Topoisomerase inhibitors, such as camptothecin, can also activate NF-κB, presumably through the generation of DSBs (Piret and Piette 1996). Although NF-κB activation in response to low-dose radiation could be due to production of reactive oxygen species (ROS; Mohan and Meltz 1994), such a model seems inconsistent with a requirement for ATM in this process.

Activation of IKK following treatment with topoisomerase inhibitors is dependent on the zinc finger domain in NEMO (Huang et al. 2003). A recent report showed that modification of NEMO with the small ubiquitin-like modifier (SUMO-1) plays a mechanistic role in the activation of IKK following treatment with topoisomerase inhibitors (Huang et al. 2003). Sumolation of NEMO may be necessary for the recruitment of NEMO to the nucleus resulting in ATM-dependent ubiquitination of NEMO and subsequent activation of IKK. Other studies have suggested that RIP is required for the activation of IKK in response to DNA damage in a manner that is independent of TRAF family members (Hur et al. 2003). It is unclear how these data can be reconciled with the proposed role for SUMO-1 in this process.

NF-κB has also been shown to be activated following UV-C irradiation; however, this response is not dependent on DNA damage (Devary et al. 1993). NF-κB activation following UV-C irradiation has been shown to be independent of IκBα Ser 32/36 phosphorylation and is, therefore, IKK-independent (Li and Karin 1998; Kato et al. 2003; Tergaonkar et al. 2003). Instead, it might operate through activation of p38 pathways leading to the activation of casein kinase 2 (CK2). CK2 then directly phosphorylates IκBα on C terminus serine residues. Whereas mutation of these serines abolishes the
ability of CK2 to induce IκB degradation following UV irradiation [Barroga et al. 1995; Lin et al. 1996; McElhinney et al. 1996; Kato et al. 2003], some early transient activation of NF-κB appears intact in CK2 knockdown cells, p38 knockout cells, or IκBα knockout cells reconstituted with IκBα in which the CK2 phosphoacceptor sites were mutated [Kato et al. 2003]. A similar pathway of NF-κB activation has been proposed in cells treated with doxorubicin [Tergaonkar et al. 2003]. Doxorubicin's mechanism of action is multiparate, and therefore it is still unclear whether the NF-κB activation observed is caused by release of free radicals and ROS via effects similar to UV-C, or caused by topoisomerase inhibition mediated through DNA intercalation. More recently, these findings have been called into question by work showing that doxorubicin and UV-C induce apoptosis through release of NF-κB dimers that can suppress transactivation of anti-apoptotic genes by recruiting histone deacetylases [Ashikawa et al. 2004; Campbell et al. 2004]. It is not clear whether these findings are generally applicable or are cell-type-specific. In summary, whereas nonlethal UV-C irradiation leads to activation of CK2 downstream from ROS, and consequent degradation of IκBα, the response to DNA damage is mediated through ATM, and perhaps RIP, to cause IKK activation. In the future, clarification of these issues will require a systematic investigation in multiple cell types using carefully controlled induction of various types of cell stress. In particular, the issue of ROS effects on NF-κB signaling merits detailed examination, given that this is likely the source of much confusion in the investigation of multiple NF-κB signaling pathways [Zhang and Chen 2004].

Regulation of IKK

With the possible exception of CK2, the common feature of all pathways leading to the activation of NF-κB is the activation of one of the IκB kinases. Consequently, this is the most important regulatory step in determining the NF-κB response to a given stimulus. Current evidence points toward a mechanism that depends on the assembly of complex signalosomes for the activation of IKK where the key event is induced proximity allowing cross-phosphorylation between the IKK subunits. However, a role for an activating IKK kinase cannot be completely ruled out. In fact, activation of IKKa in the alternative pathway by NIK is well established. Determining whether such an event also occurs in the activation of the classical pathway will require a more comprehensive analysis of the composition of the IKK complexes in various tissues and cells under different physiological conditions.

Structure and function of the IKK complex

The initial efforts to identify the IκB kinase led to the purification of a high-molecular-weight kinase complex from unstimulated cells that was capable of phosphorylating IκBα on the appropriate serine residues [Chen et al. 1996; Lee et al. 1997]. Subsequently, multiple groups identified a stimulus-dependent kinase activity that was termed the IκB kinase [DiDonato et al. 1997; Mercurio et al. 1997; Woronicz et al. 1997; Zandi et al. 1997]. The first component of this 700–900-kDa complex was shown to be CHUK, a serine/threonine kinase that, remarkably, had been proposed to be a regulator of gene transcription based solely on sequence homology [Connelly and Marcu 1995]. CHUK, a 745-amino acid protein, has since been renamed IKKa. IKKB was discovered shortly thereafter based on sequence homology and biochemical purification. IKKa and IKKB are serine/threonine kinases that are characterized by the presence of an N-terminal kinase domain, a C-terminal helix–loop–helix (HLH) domain, and a leucine zipper domain [Fig. 1]. NEMO (also known as IKKγ, IKKAP1, and Fip-3), the regulatory subunit of the IKK complex, was initially isolated through genetic complementation cloning in an NF-κB-unresponsive cell line, and subsequently by affinity purification and as a factor interacting with an adenoviral inhibitor of NF-κB [Rothwarf et al. 1996; Yamaoka et al. 1998; Y. Li et al. 1999; Mercurio et al. 1999]. NEMO is a 48-kDa protein that is not related to IKKa and IKKB and contains a C-terminal zinc finger-like domain, a leucine zipper, and N-terminal and C-terminal coiled-coil domains [Fig. 1].

IKKa and IKKB share 52% overall sequence identity and 65% identity within the catalytic domain. Both IKKa and IKKB can be inactivated through the mutation of a single lysine, Lys 44, within the predicted ATP-binding site [Fig. 1; Mercurio et al. 1997; Woronicz et al. 1997; Zandi et al. 1997]. IKKα and IKKB are capable of phosphorylating multiple members of the IκB family at multiple sites. These kinases also have substrates outside of the IκB family, most notably p65. In vitro studies indicate that the IKKa/IKKB heterodimers have higher catalytic efficiency than either homodimer [Huynh et al. 2000]. Although IKKa and IKKB display different specificities for different IκB family members, it is not known whether they are independently regulated. Although both IKKa and IKKB are capable of phosphorylating IκBα at Ser 32 and Ser 36, and IκBβ at Ser 19 and Ser 23, IKKa is less efficient and cannot replace IKKB in knockout studies [DiDonato et al. 1997; Mercurio et al. 1997; Regnier et al. 1997]. Both IKKa and IKKB are less efficient kinases for IκBβ than for IκBα, and this difference may explain the delayed degradation kinetics of IκBβ following stimulation with T NFα [Wu and Ghosh 2003]. IκBα bound to NF-κB is a better substrate for IKKβ than free IκB, which may prevent the degradation of newly synthesized IκB, thus allowing it to accumulate and down-regulate NF-κB activity [Zandi et al. 1998]. It is unclear, however, how the enzymology of the IKKs is affected by heterodimerization and complex formation in vivo.

IKKa and IKKB dimerization is dependent on the leucine zipper domain, which is therefore required for kinase activity [Mercurio et al. 1997; Woronicz et al. 1997; Zandi et al. 1997]. Available data indicate that IKKa and IKKB preferentially form heterodimers in vivo. However,
the sequence features that determine this preference have not been elucidated. Studies using knockout cells show that both IKKα and IKKβ are capable of homodimerization, although evidence for homodimers in unmanipulated cells is more controversial. Immunodepletion studies have demonstrated the presence of IKKβ/NEMO complexes activated by anti-CD3/CD28 in primary T cells [Khoshnan et al. 1999]. An IKK-only complex was also observed in HeLa cells by affinity purification, anion exchange chromatography, and immunodepletion, although this complex appeared to be only weakly activated by TNFα [Mercurio et al. 1999]. Also, because in the alternative pathway, phosphorylation of p100 in response to BAFF or LTβ stimulation can occur in the absence of IKKβ and NEMO [Senftleben et al. 2001a], it has been suggested that there is a discrete IKKα-only complex. However, there is not yet definitive evidence that IKKα homodimers, which are not bound to NEMO, exist in vivo and mediate signaling via the alternative pathway.

The HLH domain is required for full IKKβ activity and is also involved in the down-regulation of kinase activity. Deletion mutants lacking the HLH domain have diminished kinase activity following overexpression or stimulation [Delhaye et al. 1999]. Interestingly, a C-terminal deletion mutant of IKKβ [1–559] can be functionally rescued through the coexpression of the HLH domain of IKKβ [amino acids 558–756], indicating that a direct interaction between the HLH domain and kinase domain is necessary for activity. However, when the C-terminal domain of IKKβ is expressed in trans, or when the C-terminal serine residues are mutated, IKK activity is prolonged. More recent data have shown that removing the HLH region also abrogates the binding of IKK to NEMO, and hence the role of the HLH domain may be in facilitating the assembly of functional IKK–NEMO complexes [May et al. 2002].

IKKα and IKKβ interact with NEMO through a C-terminal hexapeptide sequence [Leu-Asp-Trp-Ser-Trp-Leu] termed the NEMO binding domain [NBD; Fig. 1; May et al. 2000, 2002]. A peptide encompassing this sequence when coupled to the cell-permeable antennapedia domain blocks NF-κB signaling by disrupting the IKK complex [May et al. 2000, 2002]. NEMO binding to IKK requires residues 135–231, located within the first coiled-coil motif, and can interact with the NBD of both IKKα and IKKβ. Although in vitro assembly of the complex indicates that only IKKβ assemblies with NEMO, data from IKKβ knockout mice clearly indicate otherwise, as IKKα/NEMO complexes are readily formed [Rothwarf et al. 1998; Yamaoka et al. 1998; Mercurio et al. 1999]. Competition experiments using the NBD peptide indicate that IKKβ binds to NEMO with considerably higher affinity [May et al. 2002]. Furthermore, IKKα is less sensitive to mutation of two residues within the NBD [Asp 749 and Trp 742] that abolish IKKβ binding to NEMO, indicating that the binding of IKKα is less stringent. Although IKKα or IKKβ produced in baculovirus or yeast systems is catalytically active, association with NEMO is required for in vivo IkB phosphorylation. In fact, ex-change of C-terminal portions of IKKα and IKKβ, which contain the NBD and HLH domain, confers some increase in IKKβ-like behavior on IKKα [Kwak et al. 2000]. Taken together, these results indicate that the C-terminal portion of IKK, either through the interaction with NEMO or through inducing a conformational change, strongly affects kinase activity and specificity.

NEMO has been shown to form tetramers in vitro, and it appears that NEMO can be detected in several oligomeric states in vivo [Tegelhoff et al. 2003]. In contrast, it has been reported recently that NEMO multimerization is cell-type-specific, with NEMO existing as dimer, trimer, or both, but never as tetramer [Israel 2004]. This discrepancy is likely attributable to differences in experimental systems, such as the use of different homofunctional cross-linking agents. It has also been argued that NEMO exists as a monomer in resting cells and undergoes regulated trimerization—NEMO/IKKα/IKKβ → NEMO/IKKβ [Israel 2004]. Upon trimerization, the second coiled-coil domain (CC2, amino acids 248–291) and leucine zipper domain (LZ, amino acids 294–341) align in an antiparallel fashion with an overall structure similar to the HIV-1 gp41 ectodomain [Agou et al. 2004]. When recombinant NEMO peptides encompassing the LZ and CC2 domains are mixed in vitro, they self-assemble into a pseudohexameric state. These results are consistent with previous reports that oligomerization of NEMO may occur through association of the C-terminal domain with RIP, and that forced oligomerization of NEMO activates IKK [Poyet et al. 2000]. Indeed, peptide mimics of CC2 and the LZ domain have been found to inhibit NEMO oligomerization and NF-κB activation without interfering with NEMO binding to the IKKs [Israel 2004].

Thus far, IKKα, IKKβ, and NEMO are the only components that have been conclusively demonstrated to be a part of the IKK complex. The stoichiometry of these components in the complex continues to be debated, although many researchers have supported the idea of a heterodimer of IKKα and IKKβ associating with NEMO [Rothwarf and Karin 1999; Miller and Zandi 2001]. This debate about the composition of the complex has been driven by the discrepancy between the apparent molecular weight observed during gel filtration chromatography (700–900 kDa) and the predicted size based on the actual molecular weight of the components (~200–350 kDa). There are, however, certain difficulties in defining new components of the IKK complex with any certainty. One problem has been the assumption that there is only one IKK complex. Most studies have, therefore, not made efforts to distinguish whether all or only a subset of the IKK complexes contain a given constituent. Partial purification efforts have identified proteins that likely associate only with a small subset of IKK, if at all, and may do so in a tissue-specific manner. Therefore, the only definitive components of the complex remain IKKα, IKKβ, and NEMO.

Perhaps not surprisingly, various members of the NF-κB and IκB family can associate with the IKK complex [Cohen et al. 1998; Heilker et al. 1999; Bouwmeester et
al. 2004). It has been shown more recently that the chaperone HSP90/Cdc37 is constitutively associated with the IKK complex (G. Chen et al. 2002). A role for HSP90 in IKK was supported by functional data generated using the HSP90 inhibitor geldanamycin (GA), which inhibits activation of IKK by TNF-α. HSP90, however, can associate with multiple other kinases that are involved in the NF-κB pathway, including RIP, MEKK3, MEKK1, NIK, AKT, and TBK1 (Fisher et al. 2000; Sato et al. 2000; Goos and Martin 2001). It has also been speculated that through binding to NEMO, HSP90 or HSP-70 may regulate NEMO’s oligomeric state [Agou et al. 2002, G. Chen et al. 2002]. Therefore, it is difficult to ascribe a specific role for HSP90/Cdc37 as a bona fide component of the IKK complex. Instead, HSP90 may function as a chaperone during assembly of the complex or during its regeneration following signaling.

ELKS is a 105-kDa regulatory protein that has recently been proposed to be an IKK-interacting protein [Sigala et al. 2004]. ELKS was copurified with IKKβ by immunooaffinity purification, and has structural motifs reminiscent of NEMO with coiled-coil and leucine zipper domains. In unstimulated cells, ELKS appears to be a stoichiometric component of the IKK complex based on immunodepletion experiments. Knock-down of ELKS using RNAi blocks the early activation of NF-κB following TNFα or IL-1 stimulation. However, it appears that the late phase of NF-κB activation may be intact in these cells. Furthermore, it was shown that when ELKS is knocked down, the IKK complex fails to associate with Iκκα suggesting that ELKS mediates interaction of the IKK complex with Iκκα but not Iκκβ [Sigala et al. 2004]. Therefore, it is tempting to speculate that ELKS provides the link between MEKK3, as discussed below, and assembly of the IKK/Iκκα complex necessary for activation of the early NF-κB response. Surprisingly, whereas overexpression of full-length ELKS does not activate IKK, expression of an N-terminal deletion mutant of ELKS does. The biological significance of this regulatory component has not yet been verified genetically.

There are significant emerging data arguing against the existence of additional components for the IKK complex. Recombinant NEMO when mixed with either Iκκα or Iκκβ can self-assemble into a complex of apparent molecular weight that is similar to the purified complex [Krapfmann et al. 2000]. Coproduction of NEMO with Iκκα and Iκκβ in yeast also yields an appropriately sized complex [Miller and Zandi 2001]. This may be because NEMO has a large Stokes’s radius, which explains why NEMO trimers migrate with an apparent molecular mass of 550 kDa during gel filtration chromatography [Agou et al. 2004]. Alternatively, this may simply reflect an Iκκαβ/NEMO complex of higher-order stoichiometry—a dimer of trimers. An additional IKK complex, of an apparent molecular mass of ~300 kDa has been reported by multiple groups [Zandi et al. 1997; Yamaoka et al. 1998]. This complex seems to consist of only Iκκα and Iκκβ [Yamaoka et al. 1998; Zandi et al. 1998]; however, the physiological role of this IKK complex remains to be determined.

**Genetic analysis of the role of IKK subunits**

Knockouts of each of the three IKK subunits have been generated. The IKKβ knockout phenotype is similar to that observed for the p65 knockout, supporting an argument for a central role for IKKβ in mediating NF-κB signaling via TNFα [Q. Li et al. 1999b, Z.W. Li et al. 1999, Tanaka et al. 1999]. Surprisingly, even ikκα−/− heterozygous animals have decreased NF-κB activity [Z.W. Li et al. 1999]. The similarity of their phenotypes and the ability to rescue both by knocking out TNRF1 demonstrate that IKKβ is required for TNFα signaling to NF-κB and the inhibition of TNF-induced apoptosis [Z.W. Li et al. 1999; Sentileben et al. 2001b]. Of note, TNFR/IKKβ double knockouts show a more pronounced defect in innate immune responses to bacterial infection, only surviving a few days after birth, whereas the p65 knockouts can survive for many months. These data indicate that there is no partial compensation for IKKβ by Iκκα or any other kinase.

The ikκα−/− mice are born live, but die perinatally from multiple morphological defects, particularly in epidermal and skeletal development [Hu et al. 1999; Q. Li et al. 1999a; Takeda et al. 1999]. In contrast to the ikκβ−/− mice, ikκα−/− mice do not exhibit liver apoptosis, and cells derived from these mice have only mildly reduced NF-κB activation in response to TNFα. These initial characterizations indicated that IKKα had little role in NF-κB signaling, and perhaps had some unrelated role in development, as knockout mice exhibit altered limb bud morphology at E12.5 [Q. Li et al. 1999a; Takeda et al. 1999]. The epidermal layer of the skin in Iκκα-deficient mice fails to differentiate properly, and they exhibit multiple skeletal abnormalities. Subsequent analysis has shown that these defects are NF-κB-independent. The ikκα−/− mouse can be rescued by knocking in an Iκκα in which both of the activation loop serines are mutated to alanines [IKKαAA, Cao et al. 2001; Hu et al. 2001], indicating that the role of Iκκα in epidermal development is independent of its kinase activity. More recently, conditional expression of Iκκα or kinase dead Iκκα in basal keratinocytes has been shown to be sufficient to rescue the morphogenetic defects observed in the Iκκα−/− mice [Sil et al. 2004]. The mechanism of NF-κB-independent control of keratinocyte differentiation by Iκκα is unknown; however, it is dependent on the presence of a previously unrecognized NLS in Iκκα (amino acids 232–240; Sil et al. 2004). IKKα/β double-knockout mice show further defects in NF-κB signaling. The mice are characterized by failure of neurulation during development, and MEFs from double-knockout embryos do not respond to TNFα, IL-1, or LPS [Q. Li et al. 2000]. Interestingly, the neurulation defect is not found in NEMO-deficient mice.

NEMO, like IKKβ, is required for signaling through the classical NF-κB pathway. Studies using deletion mutants of NEMO demonstrate that the C-terminal portion is required for stimulus-induced activation of IKK through interaction with upstream adapters, whereas binding to Iκκα and IKKβ occurs using sequences from
Mechanism of IKK activation

Activation of the IKK complex is dependent on phosphorylation, and can be decreased by phosphatase treatment of IKK purified from activated cells [DiDonato et al. 1997]. Most research has focused on the phosphorylation events on IKKβ, as the importance of IKKα has been appreciated more recently. IKKβ is phosphorylated on two sites within the activation loop of the kinase domain: Ser 177 and Ser 191 [Delhase et al. 1999]. IKKβ is also phosphorylated outside of the activation loop at multiple serines that are C-terminal of the HLH motif, most likely through autophosphorylation following activation. Deletion mutants that lack the C-terminal serine-rich segment are activated normally in response to TNFs but remain active for longer periods of time, indicating that autophosphorylation or trans-autophosphorylation of IKKβ is a negative regulatory mechanism [Delhase et al. 1999].

Multiple upstream kinases have been suggested to act as IKK kinases [IKK-K]. However, most such candidates failed to survive the test of gene knockouts. Such accumulation of negative results has led to the increasing belief that the IKK complex is primarily regulated through trans-autophosphorylation. Indeed, many of the proposed IKK-Ks have been found to play the role of adaptors instead of kinases. Trans-autophosphorylation caused by induced proximity and/or conformational change is an appealing mechanism of IKK activation and could be mediated through recruitment of the IKK complex by multimeric adapter/scaffolding complexes such as RIP or TRAF family members [Delhase et al. 1999; Inohara et al. 2000; Poyet et al. 2000; Tang et al. 2003]. To date, however, these studies have relied on overexpression of IKK components that may have altered the stoichiometry of the IKK complex. Therefore, the role of IKK-K or autophosphorylation must be evaluated on a stoichiometry of the IKK complex. Therefore, the role of the IKK complex following stimulation, and is required for the assembly of the active IKK complex with IκBα/IKKβ leading to the early phase of NF-κB activation (Schmidt et al. 2003). However, it is not yet clear whether MEKK3 directly phosphorylates IKK following TNF stimulation or mediates docking of IKK with IκBα/NF-κB through phosphorylation of another substrate, such as ELKS, or by acting directly as a scaffold. In this scenario it has been proposed that MEKK3 mediates assembly of IKK/IκBα complexes, whereas late stage signaling, which depends on IκBβ degradation, is mediated by MEKK2-dependent assembly of IKK/IκBβ complexes (Schmidt et al. 2003). This may represent another regulatory step through which active IKK complexes might be targeted to the appropriate IκB/NF-κB complexes.

Another MAP3K with a mysterious role in IKK activation isTpl2 [also known as Cot, MAP3K8]. Tpl2 is a serine/threonine kinase that when overexpressed can activate IKK, ERK, JNK, and p38 pathways. Expressed Tpl2 can also associate with IKKα and NIK [Lin et al. 1999]. One report implicated Tpl2 in T-cell signaling to NF-κB downstream of Akt, a serine/threonine kinase that has also been implicated in NF-κB activation [Kane et al. 2002]. Tpl2 has also been shown to interact with p105, and to induce p105 processing when overexpressed [Belich et al. 1999]. More recently it has been shown that there exists a reciprocal relationship between Tpl2 and p105 such that p105 regulates Tpl2 activity and stability [Beinke et al. 2003; Waterfield et al. 2003]. Overall, the
The relationship between Tpl2 and upstream components of the NF-κB signaling pathway remains unclear. Tpl2-deficient mice are resistant to the induction of shock by LPS administration that is independent of NF-κB-induced TNF transcription (Dumitru et al. 2000) because of a failure to activate ERK1/2, resulting in a post-transcriptional block in TNFs production (Dumitru et al. 2000). Tpl2-deficient macrophages likewise fail to up-regulate COX-2 following LPS treatment because of a decrease in CREB activity downstream of ERK1/2, Msks1, and p90RSK (Eliopoulos et al. 2002). The induction of NF-κB by LPS in Tpl2-deficient mice, however, is relatively normal. Therefore, it remains to be seen whether the interaction between Tpl2 and IKKα/NIK is of biological consequence, probably by examining other NF-κB signaling pathways in Tpl2-deficient cells.

The atypical protein kinase C (aPKC) proteins PKCζ and PCKA have also been implicated as potential IKK-Ks (Lallena et al. 1999; Sazan et al. 2000). However, these data are complicated by the observed interactions of aPKCs with RIP and TRAFs, and in the case of PKCζ, by evidence that this kinase directly phosphorylates p65 on Ser 311 (Durán et al. 2003, 2004). Surprisingly, the PKCζ−/− mice demonstrate that although PKCζ is not required for activation of IKK in MEFs, it is required in the lungs of the adult animal [Leites et al. 2001]. Therefore, it appears that the role of aPKCs in IKK activation is cell-type specific, and requires further characterization.

Akt-1 (CIKS) has been proposed to cause IKK activation [X. Li et al. 2000; Mauro et al. 2003]. Akt-1 was reported to interact with TRAF6/TRAF3 and NEMO, and consequently it may have a role in signaling from the IKK complex [Kanamori et al. 2002; Qian et al. 2002]. However, Akt−/− mice have recently been made, and exhibit splenomegaly and lymphadenopathy caused by massive B-cell expansion, possibly from increased Bcl-XL levels [Li 2004]. The B cells in these mice display increased NF-κB and AP-1 responses following CD40 ligation, indicating the function of Akt-1 is quite different from previously thought. Stimulation of cells by dsRNA activates NF-κB signaling through TLR-dependent and TLR-independent mechanisms. The dsRNA-responsive protein kinase PKR is induced by IFNγ and is activated by viral dsRNA, resulting in a blockade of protein synthesis through phosphorylation of eIF-2α (for review, see D’Acquisto and Ghosh 2001). Activation of IKK and JNK are also an important component of the antiviral response through PKR [Kumar et al. 1994; Chu et al. 1999]. Activation of NF-κB by PKR is probably mediated through a direct interaction with IKK, most likely IKKβ, but is independent of NEMO, and is independent of PKR kinase activity [Bonnet et al. 2000; Ishii et al. 2001]. Therefore, like RIP and IRAK, PKR probably serves as a scaffold in this NF-κB signaling pathway.

As discussed above, autophosphorylation of the C-terminal domain of the IKKs is likely to play an important role as part of a negative feedback loop. Degradation of IκB may allow IKK to phosphorylate the C-terminal serines due to decreased substrate competition. Phosphorylation of the C-terminal domain of IKK may recruit a phosphatase capable of dephosphorylating the activation-loop serines, such as protein phosphatase-2Cβ [Prajapati et al. 2004]. Supporting evidence for such a model comes from experiments where treatment of cells with the phosphatase inhibitor okadaic acid leads to increased NF-κB activity and IκB degradation [DiDonato et al. 1997; Miskolci et al. 2003]. However, it is difficult to determine unequivocally whether the phosphatases act directly on the phosphorylated IKKs or on other pathway components.

A widely studied negative regulator of NF-κB is A20. A20 is an NF-κB-regulated gene, containing a zinc finger and TRAF-binding domain, that is expressed rapidly following TNFα induction [Kikos et al. 1992; Laherty et al. 1992]. A20 has been shown by multiple groups to inhibit TNFα induction of NF-κB [Cooper et al. 1996; Jättel et al. 1996; Song et al. 1996]. It appears that A20 inhibits NF-κB transcriptional activity independent of nuclear localization, and it is unclear if A20 affects the activity of IKKβ or blocks IκB degradation [Zetoune et al. 2001]. Interestingly, A20 interacts with IKKα but not with IKKβ, and thus may provide a means to negatively regulate NF-κB activity downstream of IκB degradation [Zetoune et al. 2001]. A20-deficient mice exhibit an inflammatory phenotype and increased sensitivity to TNF and LPS, and cells from these mice show prolonged NF-κB activation in response to TNF [Lee et al. 2000]. A20 might also act indirectly by blocking IKK and thereby allowing newly synthesized IκBα to accumulate, translocate to the nucleus, and remove NF-κB-bound to DNA [Song et al. 1996; Zhang et al. 2000]. A20 has also been recently reported to be a deubiquitinating enzyme (DUB), although the significance of this function is unknown [Evans et al. 2004].

There is increasing interest in the role of nonphosphorylation modifications in the regulation of signaling pathway components (for review, see Lynch and Gadina 2004). In particular, recent studies have shown that multiple components of NF-κB signaling pathways act as ubiquitin ligases, substrates, or both [Ben-Neriah 2002]. There has been tremendous interest recently in the role of regulatory ubiquitination, particularly Lys 63-linked monoubiquitination, in signaling to NF-κB. Members of the TRAF family have E3 ligase activity, and have been proposed to catalyze an auto ubiquitination necessary for activation of IKK downstream of TNF signaling. Genetic evidence for the role of ubiquitin in TNF signaling comes from investigations of the cylindromatosis gene product, CYLD, which has been proposed to serve as a deubiquitinating enzyme for TRAF2 [Brummelkamp et al. 2003; Kovalenko et al. 2003; Trompouki et al. 2003]. Hence, CYLD may negatively regulate NF-κB activation by TNF family members, although how ubiquitination of TRAF2 participates in activation/recruitment of the IKK complex is not clear. It is assumed that an ubiquitination event might be required for the aggregation of the IKK complex, but as yet, no clear mechanism to explain such a connection exists. The protein cIAP1 has
been shown to ubiquitinate NEMO, and perhaps the interaction between NEMO and cIAP1 is mediated by ubiquitinated TRAF2 (Tang et al. 2003). Ubiquitination by cIAP1 has been proposed to cause TRAF2 degradation, which is surprising because Lys 63 ubiquitination, which is mediated by Ubc13 and MMS2, is a regulatory event and is the target of CYLD DUB activity. It is therefore not yet clear whether the ubiquitination of NEMO directly leads to activation of the IKK complex. It is tempting to speculate that ubiquitination of NEMO might result in the formation of NEMO oligomers that can mediate IKK activation through conformational change and autophosphorylation.

TRAF6 ubiquitin ligase activity has recently been shown to be able to activate IKK in cell lysates (Sun et al. 2004). In the Toll/IL-1 signaling pathway, biochemical studies implicated the ubiquitin ligase activity of TRAF6 (Deng et al. 2000). K63-linked autoubiquitination of TRAF6 was shown to be required for association with and activation of TAK1. The TAK1 complex has previously been proposed to preassemble on IRAK family members, via an interaction between IRAK and TAB2 [Takaesu et al. 2000]. More recently it has been argued that the Lys 63 polyubiquitin chain on TRAF6 is bound by the zinc finger domain of TAB2, resulting in activation of TAK1 [Chen 2004; Sun et al. 2004]. It is not known if there is a DUB responsible for the negative regulation of this signaling pathway in a manner analogous to that observed for CYLD.

The small ubiquitin-like modifier SUMO-1 has also been suggested to play a role in NF-κB signaling. Sumolation of IκBα occurs in multiple cell types and is not affected by stimulation [Desterro et al. 1998]. IκBα contains a target sequence for sumolation between amino acids 19 and 25, and can be sumolated by Ubch9 in vitro, primarily on Lys 21. Overexpression of SUMO inhibits NF-κB activation by multiple stimuli, indicating that SUMO modification serves to prevent ubiquitination of a subfraction of IκBα. Regulation of SUMO levels may, therefore, dictate the amount of NF-κB that can be activated. A more recent report [already discussed above] implicated sumolation of NEMO in the response to genotoxic stress. Sumolation was suggested to cause NEMO nuclear localization, and the nuclear NEMO was then subjected to ubiquitination. It is currently unknown, however, how genotoxic stress leads to this modification of NEMO.

Regulation of NF-κB transcriptional activity

The activity of NF-κB is also regulated through covalent modifications that alter the ability of the NF-κB dimers to bind DNA, recruit coactivators to the enhancer region, and interact with IκB. Stimulus-dependent phosphorylation of p65 was the first regulatory event that was recognized to occur downstream from IκB degradation [Naumann and Scheidereit 1994; Neumann et al. 1995]. Both p65 and c-Rel have a consensus protein kinase A (PKA) site at Ser 276, located ~25 residues N-terminal to their NLS, and overexpression of PKA leads to increased DNA binding activity [Fig. 4A; Mosialos and Gilmore 1993; Neumann et al. 1995]. The involvement of PKA in regulation of NF-κB was further enhanced with the finding that the catalytic subunit of PKA exists in a complex with cytosolic NF-κB–IκB complexes [Zhong et al. 1997]. Degradation of IκB upon stimulation activates this associated PKAc, which then phosphorylates p65 on Ser 276. However, it has also been reported that in certain cells the cytosolic p65 is already phosphorylated on Ser 276 before stimulation, suggesting that phosphorylation might occur concomitant with the assembly of the cytosolic NF-κB–IκB–PKAc complex [Anrather et al. 1999]. The role of this phosphorylation...
appears to be twofold. First, the phosphorylation is necessary for enhancing the efficiency of DNA binding by p65, and second, it provides an additional interaction site for the transcriptional coactivator CBP/p300 [Fig. 4B]. In unphosphorylated p65, the C-terminal region of the protein interacts with the Rel-homology domain, thereby interfering with DNA binding as well as association with CBP/p300. Phosphorylation of Ser 276 prevents this intramolecular association between the N- and C-terminal domains of p65 and therefore facilitates both DNA binding and transcriptional activity of NF-κB [Zhong et al. 1998, 2002]. Recently the protein kinases MSK1 and MSK2 [mitogen and stress activated protein kinase], which possess substrate specificity identical to PKA, were also shown to phosphorylate Ser 276 and stimulate NF-κB transcriptional activity (Vermeulen et al. 2003). Because MSK1/2 and PKA have identical substrate specificity, the only difference appears to be in their subcellular localization. MSK1/2 are nuclear, whereas the NF-κB:IκB-associated PAKα is believed to be in the cytosol. Because the MSK1<sup>−/−</sup>, MSK2<sup>−/−</sup> MEFs seem to demonstrate significantly diminished transcriptional activity in response to TNF, it appears that the MSK kinases contribute to the phosphorylation of Ser 276, although how these nuclear kinases synergize with cytosolic PKA in phosphorylating nuclear NF-κB remains to be clarified (Vermeulen et al. 2003).

Additional p65 phosphorylation events have been described, although their significance remains unclear. IKK is one of the many kinases implicated in the direct phosphorylation of p65 at Ser 536 [Sakurai et al. 2003]. A recent study showed that IKKα was responsible for phosphorylation of p65 at Ser 536, and that this event was required for transcriptional activation following introduction of the HTLV Tax gene into MEFs [O'Mahony et al. 2004]. A role for IKKβ in regulating the transcriptional activity of NF-κB by phosphorylating p65 was also suggested in a previous study that examined activation of NF-κB in response to PI-3K/Akt pathway, although the actual sites for phosphorylation were not tested [Sizemore et al. 2002]. Surprisingly, another recent study demonstrated that IKKγ, but not IKKα, was responsible for phosphorylating p65 at this same site following LPS stimulation [Yang et al. 2003]. This confusion also extends to potential phosphorylation at another serine residue at position 529. It has been reported that this site is phosphorylated by CK2 following IL-1 or TNF-α stimulation [Bird et al. 1997; Wang and Baldwin 1998; Wang et al. 2000], although there is less evidence that it regulates the NF-κB transcriptional response [Yang et al. 2003]. It is even less clear whether such phosphorylation events can be generalized to other members of the Rel family. Indeed, it is likely that each member of the Rel family of proteins has distinct requirements for modification that affect transcriptional responses, adding more complexity and specificity to post-IκB regulation of NF-κB pathways.

TNFα signaling to NF-κB also regulates the transcriptional activity of NF-κB through direct modification of p65. Akt and members of the atypical protein kinase C (PKC) family have been implicated in this process, as have the IKKs. RIP1 has been proposed to link TNFR1 to PKCζ via p62 [Sanz et al. 1999, 2000], and multiple studies have reported the ability of PKCζ to phosphorylate p65 at Ser 311 [Anrather et al. 1999, Leitges et al. 2001]. Furthermore, NF-κB DNA binding is drastically decreased despite normal IKK activation in PCKζ<sup>−/−</sup> MEFs. The link between PKCζ and p65 phosphorylation requires further characterization.

Besides the protein kinases that are capable of phosphorylating specific sites on p65 such as PKA, PKCζ, CK2, and IKKα/β, two other kinases have been implicated in NF-κB regulation through genetic studies, although it is unknown whether they directly phosphorylate p65. The kinases, TBK1 (T2K, NAK), and GSK3β were first suspected of being involved in NF-κB activation because their knockout led to embryonic lethality at approximately E15 from massive hepatocyte apoptosis. Subsequent analysis with knockout MEFs showed that although degradation of IκB and nuclear translocation and DNA binding by NF-κB was normal in these cells, they were dramatically deficient in NF-κB-dependent transcriptional activity [Bonnard et al. 2000; Hoe-flich et al. 2000]. Similar results were obtained using LiCl as a GSK3β inhibitor in wild-type hepatocytes (Schwabe and Brenner 2002). However, a recent report was unable to reproduce the defect in transcription using TBK1 knockout MEFs, and therefore it is unclear what role, if any, TBK1 plays in regulating the transcriptional activity of NF-κB [McWhirter et al. 2004].

Recently an additional phosphorylation event on Thr 254 of p65 has been suggested to play an important role in regulation of nuclear NF-κB function [Ryo et al. 2003]. This phosphorylation event is reported to be the target of postphosphorylation modification by the peptidyl-prolyl isomerase PIN-1 [Ryo et al. 2003]. The authors posit that PIN-1 modifies p65 down-regulation by preventing binding of SOCS-1 (suppressor of cytokine signaling 1), and subsequent SOCS-1-mediated ubiquitination and proteasomal degradation of p65. However, as discussed by others, mice deficient in either Pin-1 or SOCS-1 do not exhibit phenotypes that would be consistent with such a prominent role in NF-κB signaling [Ben-Neriah 2003]. Yet another nuclear p65 interaction that merits further study is peroxisome proliferator-activated receptor-γ (PPARγ), a nuclear hormone receptor and transcription factor. PPARγ is reported to negatively regulate NF-κB signaling at the level of p65 as well as upstream of IκB, a role that is supported by increased NF-κB activity in PPARγ<sup>−/−</sup> heterozygous mice [Setoguchi et al. 2001; Kelly et al. 2004]. PPARγ and NF-κB are proposed to inhibit each other in a manner that results in export of both to the cytoplasm [Kelly et al. 2004].

It is probably fair to say that it is difficult to reconcile all the published data concerning the different sites of phosphorylation, and the different protein kinases, into a unified model. Interestingly, among the four proposed sites of phosphorylation, two are in the dimerization domain of the Rel-homology domain (Ser 276 and Ser 311), whereas the remaining two sites are in the C-terminal, TAD region (Ser 529 and Ser 536). It is possible that
phosphorylation on at least one site among either group is necessary for transactivation. As reported earlier, phosphorylation at Ser 276 was shown to decrease the intramolecular interaction between the C-terminal and the N-terminal domains of p65 [Fig. 4B]. If this interaction is regulated through phosphorylation, then phosphorylation at any of the sites would contribute to the unfolding of the C terminus of p65 from the N terminus, thereby unmasking the domains that interact with CBP/p300. Hence, phosphorylation at all four sites would probably be most efficient in promoting transactivation. However, this model appears inconsistent with published studies where reconstitution of p65-deficient MEFs with single-site mutants at positions 276, 311, 529, and 536 all abolish transcriptional activity. Also depending on the laboratory, cells lacking MSK1/2, PKCζ, and IKKα (cells lacking PKA and CK2 are unavailable because of early embryonic lethality) all appear to contribute equally to the transcriptional activation of NF-κB [Leitges et al. 2001; Sizemore et al. 2002; Vermeulen et al. 2003]. Clearly, further analysis will be required to resolve which phosphorylation sites are critical for NF-κB activity, and if multiple phosphorylation events all contribute to the final outcome, then it will be important to understand how the cell sorts out the contribution of the different phosphorylations. One concern that remains about techniques in which knockout MEFs are reconstituted with mutants is that the site of integration of the expression vector or retrovirus into the genome might affect some regulatory gene that contributes to NF-κB activity. Therefore, the best way to address such concerns would be to introduce mutant forms of p65 into the genome using a “knock-in strategy,” although it is unclear what would be the interpretation if such approaches also showed equivalent importance for all the proposed phosphorylation sites.

The role of direct acetylation of p65 in the regulation of transcriptional activity is even more controversial. Acetylation of p65 by CBP/p300 was initially reported to prolong NF-κB DNA binding by preventing the interaction with IκB [Chen et al. 2001]. This model posits that p65 must then be deacetylated by HDAC-3, which directly interacts with p65, before being bound by IκB and exported to the cytosol. However, a subsequent report suggested that acetylation of p65 by both CBP and p300/CBP-associated factor (PCAF) on Lys 122/123 did not change the affinity of p65 for IκB [Kienman et al. 2003]. Finally, it has also been suggested that acetylation at Lys 221 increases affinity of p65 for the κB site and inhibits IκB binding, whereas acetylation of Lys 310 enhances transcriptional activity without altering binding to DNA or IκB [L.F. Chen et al. 2002]. Although these data provide an intriguing twist to p65 regulation, which is consistent with mechanisms that regulate the activity of other transcription factors such as p53 (Gu and Roeder 1997), the physiological relevance of these findings in NF-κB remains to be established using targeted genetic approaches.

NF-κB-independent regulation of transcription by components of the NF-κB signaling pathway is also emerging as a novel regulatory step in NF-κB activation. Nuclear IKKα was recently shown to mediate the phosphorylation of histone H3. Chromatin immunoprecipitation (ChIP) experiments clearly show accumulation of IKKα, and perhaps IKKβ, at the promoter sites of NF-κB-responsive genes [Anest et al. 2003; Y. Yamamoto et al. 2003]. Furthermore, in the absence of IKK-α, gene transcription diminishes despite normal recruitment of p65 to the κB enhancer. Analysis of the histone status indicates that induced phosphorylation of H3 does not occur in the absence of IKKα but is maintained without IKKβ. It remains unclear whether IKKα catalyzes histone phosphorylation directly or through a downstream kinase and what the biological relevance of this finding is, given that classical NF-κB activity remains largely intact in IKKα−/− cells.

**Perspective and summary**

The progress in understanding the regulatory mechanisms that operate in the NF-κB signaling pathway, and understanding how NF-κB functions in normal biology and disease, has been tremendous. A search of PubMed reveals nearly 15,000 papers reporting on some facet of NF-κB, clearly a reflection of the pleiotropic nature of this transcription factor that makes it the subject of inquiry for many different laboratories with diverse interests. Although we continue to gain insight from parallel research efforts in different systems, it is fair to say that there is much that remains to be learned. Nevertheless, certain themes are clearly emerging. That IKK activation occurs through oligomerization, either of the IKK complex itself or upstream scaffolds, that ubiquitination plays a role in the activation of IKK, and that phosphorylation of p65 plays an important role are ideas that continue to gain support. However, the mechanistic details are lacking in many of these areas. One significant hurdle continues to be the lack of structural information about the IKKs, although progress continues to be made in illuminating the functional significance of structural motifs of the IKK components.

Another area of recent progress has been in understanding the relationship between NF-κB and the pathophysiology of various diseases. Abnormal NF-κB signaling is the subject of particularly intensive research focusing on cancer, inflammatory diseases, and neurodegenerative disorders. However, it remains to be seen whether specific inhibition of only NF-κB will be sufficient for treatment of these diseases or will help increase the efficacy of current treatments as an adjuvant therapeutic. Either way, the promise of NF-κB as a therapeutic target can only be fulfilled through a better understanding of the mechanistic differences that regulate specific NF-κB signaling pathways, and we are therefore likely to see continued progress at the same pace in the next few years.

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