

# Review

## New Insights into the Role of Nuclear Factor- $\kappa$ B in Cell Growth Regulation

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**The nuclear factor (NF)- $\kappa$ B family of eukaryotic transcription factors plays an important role in the regulation of immune response, embryo and cell lineage development, cell apoptosis, cell-cycle progression, inflammation, and oncogenesis. A wide range of stimuli, including cytokines, mitogens, environmental particles, toxic metals, and viral or bacterial products, activate NF- $\kappa$ B, mostly through I $\kappa$ B kinase (IKK)-dependent phosphorylation and subsequent degradation of its inhibitor, the I $\kappa$ B family of proteins. Activated NF- $\kappa$ B translocates into the nucleus where it modulates the expression of a variety of genes, including those encoding cytokines, growth factors, acute phase response proteins, cell adhesion molecules, other transcription factors, and several cell apoptosis regulators. During the past few years, tremendous progress has been achieved in our understanding on how intracellular signaling pathways are transmitted in either a linear or a network manner leading to the activation of NF- $\kappa$ B and subsequent cell growth control. However, a detailed molecular mechanism of NF- $\kappa$ B regulating cell growth has yet to be determined. Elucidation of the relationships between NF- $\kappa$ B activation and cell growth will be important in developing new strategies for the treatment of various human diseases, such as chronic autoimmune disorder and cancer. (*Am J Pathol* 2001, 159:387–397)**

After more than a decade of intensive study, a complex body of knowledge has been accumulated, revealing the molecular mechanisms of signal-induced activation of nuclear factor (NF)- $\kappa$ B, a pivotal transcription factor governing the expression of early response genes involved in cell-to-cell interaction, intercellular communication, cell recruitment or transmigration, amplification or spreading of primary pathogenic signals, and initiation or acceleration of tumorigenesis.<sup>1–3</sup> Presently, five mammalian NF- $\kappa$ B family members have been identified and

cloned.<sup>4–6</sup> These include NF- $\kappa$ B1 (p50/p105), NF- $\kappa$ B2 (p52/p100), RelA(p65), RelB, and c-Rel. All of these NF- $\kappa$ B family members share a highly conserved Rel homology domain responsible for DNA binding, dimerization, and interaction with I $\kappa$ B, the intracellular inhibitor for NF- $\kappa$ B.<sup>7</sup> The C-terminal regions of RelA, RelB and c-Rel contain a transactivating domain that is important for NF- $\kappa$ B-mediated gene transactivation. The C-termini of the precursor molecules for p50 and p52, p105 and p100, contain multiple copies of the so-called ankyrin repeat, which is found in I $\kappa$ B family members, including I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$ , I $\kappa$ B- $\epsilon$ , Bcl3, and *Drosophila* cactus.

A wide range of signals, which typically include cytokines, mitogens, environmental and occupational particles, toxic metals, intracellular stresses, viral or bacterial products, and UV light, induce expression of early response genes through the NF- $\kappa$ B family of transcription factors.<sup>2,4,8–10</sup> In resting cells, NF- $\kappa$ B is sequestered in the cytoplasm in an inactive form through its association with one of several inhibitory molecules, including I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$ , I $\kappa$ B- $\epsilon$ , p105, and p100. Activation of the NF- $\kappa$ B-signaling cascade results in a complete degradation of I $\kappa$ B or partial degradation of the carboxyl termini of p105 and p100 precursors, allowing the translocation of NF- $\kappa$ B to the nucleus, where it induces transcription (Figure 1). Activated NF- $\kappa$ B binds to specific DNA sequences in target genes, designated as  $\kappa$ B-elements, and regulates transcription of genes mediating inflammation, carcinogenesis, and pro- or anti-apoptotic reactions.

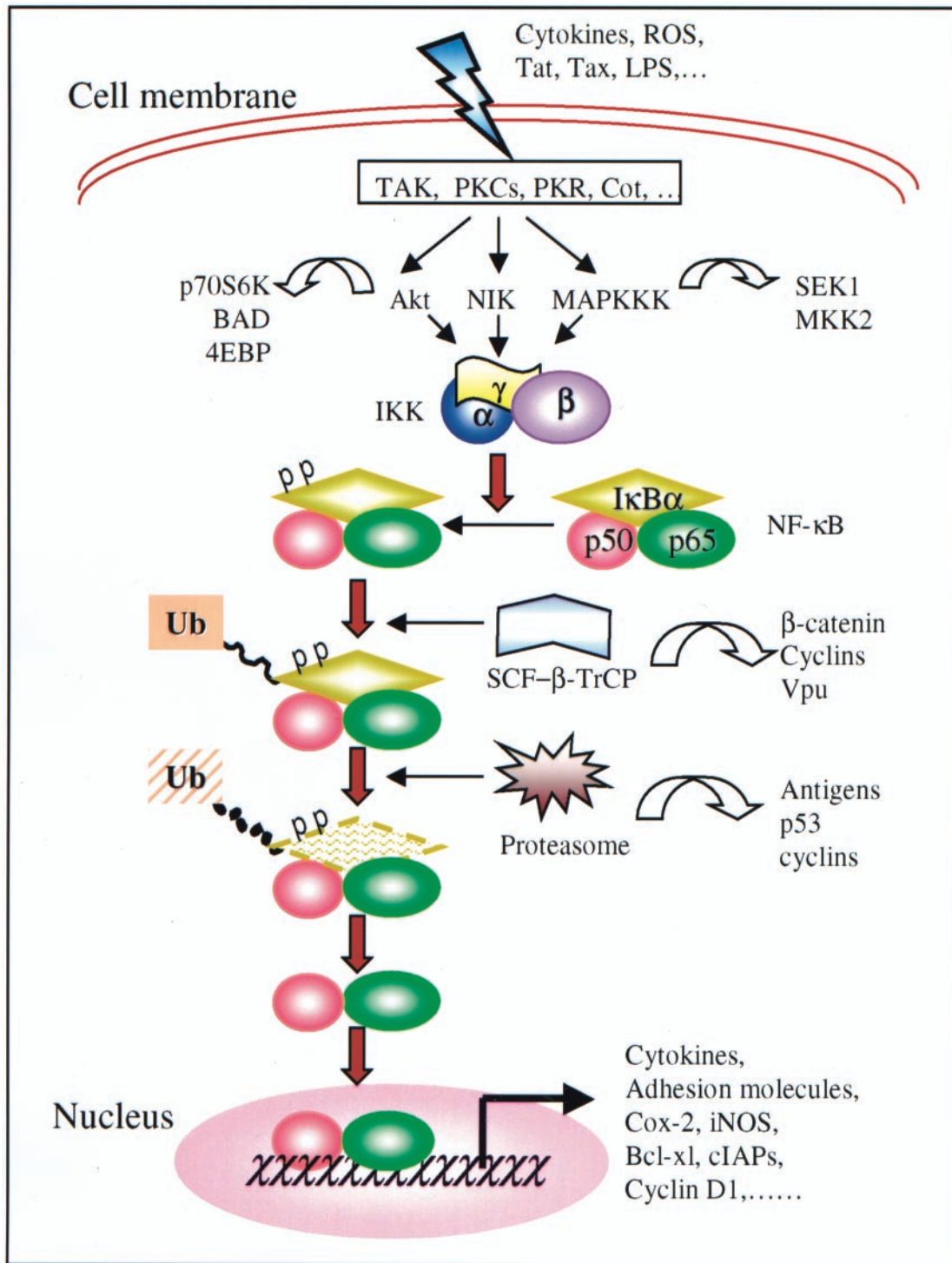
Three I $\kappa$ B proteins, I $\kappa$ B- $\alpha$ , I $\kappa$ B- $\beta$ , and I $\kappa$ B- $\epsilon$ , have been identified, among which I $\kappa$ B- $\alpha$  is the most abundant inhibitory protein for NF- $\kappa$ B.<sup>4</sup> All I $\kappa$ B proteins contain two conserved serine (S) residues within their N-terminal domain. Phosphorylation of these conserved S residues in response to inducers, leads to the immediate polyubiquitination of I $\kappa$ B proteins by the SCF- $\beta$ -TrCP complex, a step that has been shown recently to be inhibited by the nonpathogenic *Salmonella* bacteria in gut epithelial

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**Figure 1.** Signaling pathways of NF- $\kappa$ B activation. Extracellular inducers, including cytokines, reactive oxygen species (ROS), and viral and bacterial products, activate IKK through upstream kinases directly or indirectly. Activated IKK phosphorylates N-terminal S32 and S36 residues of I $\kappa$ B- $\alpha$  that is associated with NF- $\kappa$ B p50 and p65 heterodimer. The SCF- $\beta$ -TrCP complex recognizes phosphorylated I $\kappa$ B- $\alpha$  and modifies I $\kappa$ B- $\alpha$  with polyubiquitin chains. This is followed by proteasome-mediated degradation of I $\kappa$ B- $\alpha$ . After degradation of I $\kappa$ B- $\alpha$ , the activated NF- $\kappa$ B translocates into the nucleus where it binds to the  $\kappa$ B-sites of gene promoters or enhancers to up-regulate target gene expression. **Line arrows** and **filled arrows** denote the NF- $\kappa$ B signaling pathways; **open arrows** denote the connections with the by-standing signaling pathways.

cells.<sup>11</sup> This modification subsequently targets I $\kappa$ B proteins for rapid degradation by the 26S proteasome.<sup>12</sup> A high-molecular weight complex that phosphorylates I $\kappa$ B- $\alpha$  or I $\kappa$ B- $\beta$  has been characterized recently and

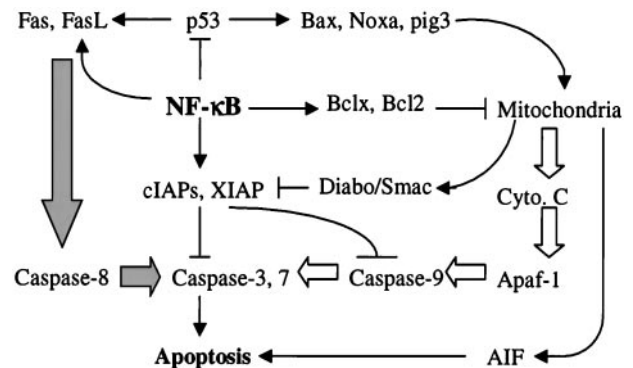
named I $\kappa$ B kinase (IKK) complex. This complex contains two catalytic subunits, IKK- $\alpha$  and IKK- $\beta$ , and a structural component named NEMO/IKK $\gamma$ /IKKAP.<sup>13,14</sup> An earlier report by Cohen and colleagues<sup>15</sup> suggested the presence

of a scaffold protein named IKK complex-associated protein (IKAP) in the IKK complex, which could not be confirmed in other studies. A later study by the group, who originally identified IKAP, indicated that the observed association of IKAP with IKK was because of a nonstrict elution condition of chromatographic extracts during the purification of IKK.<sup>16</sup> Recently, two groups independently reported the identification of a novel protein, CIKS/Act1, associated with the IKK complex and suggested that CIKS/Act1 functions as an anchoring proteins in the assembly of the IKK complex and in providing a possible connection between IKK and c-Jun-N-terminal kinase signaling.<sup>17,18</sup> IKK- $\alpha$  and IKK- $\beta$  share 50% sequence homology. Both proteins contain an amino terminal kinase domain, a carboxyl terminal region with a leucine zipper, and a helix-loop-helix domain. *In vitro* or *in vivo* studies indicate that although both IKK- $\alpha$  and IKK- $\beta$  are capable of phosphorylating I $\kappa$ B- $\alpha$  on ser32 and ser36, IKK- $\beta$  is more potent in I $\kappa$ B- $\alpha$  phosphorylation induced by proinflammatory stimuli. However, a distinct IKK complex, named IKKi/ $\epsilon$  that does not contain IKK- $\alpha$ , - $\beta$ , or - $\gamma$ , was recently identified in T cells.<sup>3,19</sup> IKKi/ $\epsilon$  shares 27% homology with IKK- $\alpha$  and IKK- $\beta$  and possibly mediates NF- $\kappa$ B-activating kinase signaling and PMA/PKC $\epsilon$ -induced S36 phosphorylation of I $\kappa$ B- $\alpha$  and NF- $\kappa$ B activation.<sup>3,19</sup>

Although the signaling pathways leading to the activation of NF- $\kappa$ B have been well defined, a number of questions remain to be answered. For example, it is unclear exactly how many protein subunits comprise a naïve and activated IKK complex, respectively; how the various signaling pathways converge on this kinase complex; what upstream kinases contribute to the phosphorylation and activation of IKK. It is also unclear whether other substrates, in addition to I $\kappa$ B or NF- $\kappa$ B family members, can be phosphorylated by IKK. Regarding the functional aspects of NF- $\kappa$ B transcription factor, we know that although NF- $\kappa$ B is important and involved in the regulation of cell apoptosis, cell-cycle transition, and carcinogenic transformation,<sup>20</sup> the detailed interconnections among NF- $\kappa$ B activation, cell cycle, and apoptosis are still undefined. Considering the fact that aberrant activation of NF- $\kappa$ B is associated with a wide range of human diseases, elucidation of molecular mechanisms determining NF- $\kappa$ B activation and expression of its various functions may lead to the development of novel preventive and therapeutic strategies for many diseases including chronic inflammation and cancer.

### Roles of NF- $\kappa$ B Activation in Cell Apoptosis

Programmed cell death, or apoptosis, is an essential mechanism for any multicellular organism to eliminate cells that are in excess or potentially dangerous.<sup>21</sup> Most apoptotic cells are characterized by unique morphological features, such as membrane blebbing, cell shrinking, cytosolic and nuclear condensation, and breakdown of chromosomal DNA. Depending on the use of different initiating caspases, signal-induced apoptosis can be roughly divided into receptor-mediated extrinsic apopto-



**Figure 2.** Possible targeting point of anti-apoptotic signals from NF- $\kappa$ B. Intrinsic (open arrows) and extrinsic (filled arrows) apoptosis pathways are depicted. The effector caspases, such as caspase-3 and caspase-7, are activated by upstream initiator caspases, caspase-8 and caspase-9. The initiator caspases themselves are activated by either ligands binding to the death receptor complex or cytochrome c released from damaged mitochondria. An anti-apoptotic effect of NF- $\kappa$ B is achieved through its up-regulation of IAPs that inhibits caspases and Bcl-xl that protects mitochondria from further damaging.  $\rightarrow$ , activation;  $-$ , inhibition.

sis and mitochondrial-mediated intrinsic apoptosis (Figure 2).<sup>22,23</sup> The extrinsic apoptotic pathway is triggered as a consequence of ligand binding to death receptors, including tumor necrosis factor (TNF)-R, Fas, OX40, CD40, and 4-1BB, which contain conserved protein-protein-binding domains termed death domains. These receptors recruit procaspases, mainly caspase-8, via adapter molecules. The intrinsic apoptotic pathway is mediated by mitochondria through release of apoptosis-promoting factors, including cytochrome c, apoptosis-inducing factor, and Diablo/Smac.<sup>24-27</sup> Cytochrome c forms a complex with a cytosolic protein named Apaf-1, a flavoprotein with homology to plant ascorbate reductases and bacterial NADH oxidases, to activate caspase-9.<sup>27</sup> Whereas apoptosis-inducing factor released from the intermembranous space of damaged mitochondria induces apoptosis in a caspase-independent manner, Smac/Diablo released from mitochondria promotes apoptosis by binding to and antagonizing XIAP, cIAP1, and cIAP2, allowing the activation of caspases.<sup>25,26</sup> Both activated caspase-8 from the extrinsic apoptotic pathway and activated caspase-9 from the intrinsic apoptotic pathway cleave and activate effector caspases, mainly caspase-3 to execute an apoptotic process. These apoptotic pathways, however, were compromised in many cases because of the activation of caspase-independent signaling cascades that function to block the apoptotic responses. A good example is TNF-R-mediated activation of NF- $\kappa$ B that induces expression of anti-apoptotic proteins, including caspase inhibitors, such as cIAP1, cIAP2, and XIAP, and mitochondria membrane stabilizers, such as Bcl-xl and Bfl-1.<sup>28-30</sup>

Tumor suppressor protein, p53, has been considered to be one of the major contributors of cell apoptosis in response to a variety of stress inducers. As a transcription factor, p53 is able to up-regulate the expression of genes involved in either reactive oxygen species production or reactive oxygen species metabolism, including quinone oxidoreductase (Fig 3), proline oxidase (Fig 6) homologues, glutathione transferase (Fig 12), and gluta-

thione peroxidase (GPx).<sup>31</sup> Moreover, p53 also activates the expression of several genes that directly control or regulate the process of apoptosis. These genes include Bax, Fas, Fas ligand (FasL), IGF-BP3, PAG608,<sup>32</sup> ei24 (Fig 8),<sup>33</sup> and Noxa.<sup>34</sup>

With the identification of role of NF- $\kappa$ B in transcriptional regulation of several pro-apoptotic genes, such as fas and fasl, controversy raged as reports demonstrating that NF- $\kappa$ B protected cells from apoptosis in some types of cells were matched by a similar number of reports demonstrating that it did not in other types of cells.<sup>35,36</sup> Based primarily on earlier studies, NF- $\kappa$ B was initially considered a pro-apoptotic factor because of its rapid activation in cells in response to apoptotic signals and its involvement in the expression of some apoptotic genes, including TNF- $\alpha$ , *c-myc*, and fasl.<sup>37,38</sup> More recent work, however, has altered this view and revealed an anti-apoptotic effect of NF- $\kappa$ B in response to a variety of apoptotic stimuli.

The direct evidence for the anti-apoptotic effects of NF- $\kappa$ B is provided by gene knockout studies in which the genes encoding either members of NF- $\kappa$ B family proteins or upstream kinases were disrupted. RelA (p65)-deficient mice die during embryonic development through apoptosis of hepatocytes.<sup>39</sup> IKK- $\beta$  gene knockout mice and IKK- $\beta$ /IKK- $\alpha$  double-knockout mice die as embryos and show massive liver cell apoptosis,<sup>40–42</sup> a phenotype similar to the response of NF- $\kappa$ B p65 gene knockout mice. In addition, knockout of the IKK- $\alpha$  gene results in perinatal lethality of mice with an increased thickness of the skin because of the deficiency of keratinocyte differentiation.<sup>43</sup> Male mice with an inactivated X-linked gene encoding IKK- $\gamma$ /NEMO, an essential modulator of the IKK complex for NF- $\kappa$ B activation, die at mid-gestation because of a massive apoptosis of cortical and medulla lymphocytes in the thymus, in addition to degeneration of the liver.<sup>44,45</sup> Female mice deficient in the IKK- $\gamma$ /NEMO gene manifest a unique dermatopathy because of the apoptosis of keratinocytes and consequent abnormal pigmentation, a characteristic strikingly similar to that of the human X-linked dominant, male-lethal genetic disease—incontinentia pigmenti or Bloch-Sulzberger Syndrome. Cross-breeding of relA or IKK- $\beta$  gene knockout mice with TNF-R1 or TNF- $\alpha$  gene knockout mice revealed partial rescue of embryonic lethality, suggesting that NF- $\kappa$ B deficiency sensitized cells in response to TNF- $\alpha$ -mediated cytotoxicity.<sup>46,47</sup> Similarly, mice deficient in both TNF-R1 and IKK- $\beta$  showed an attenuated embryonic liver apoptosis.<sup>48</sup>

Other compelling evidence linking NF- $\kappa$ B with an anti-apoptotic effect is based on the studies indicating that NF- $\kappa$ B is a priming factor for liver regeneration after partial hepatectomy.<sup>49</sup> This priming effect of NF- $\kappa$ B might be through its transcriptional regulation for survival genes or anti-apoptotic genes whose products can block stress signal-induced cell death, a process critically involved in cell proliferation and transformation. Candidate anti-apoptotic genes targeted by NF- $\kappa$ B include those encoding the cell-cycle regulatory protein cyclin D1,<sup>50–52</sup> the mitochondrial membrane-stabilizing proteins Blf-1 and Bcl-xl,<sup>53,54</sup> the caspase inhibitors cIAP1/cIAP2 and XIAP, and

the TNF receptor-associated factors TRAF1 and TRAF2.<sup>55</sup> It should be noted that several reports suggest that NF- $\kappa$ B is also a pro-apoptotic factor in FasL-induced cell death.<sup>56,57</sup> This argument is primarily based on earlier observations that NF- $\kappa$ B can regulate the artificial promoter activity of the fasl gene, a gene encoding an important activator of apoptosis through a CD95/Fas- and Fas-associated death domain (FADD)-mediated caspase-8 activation pathway.<sup>22</sup> However, both promoter truncation studies of the fasl gene and somatic cell mutagenesis studies of IKK- $\gamma$  indicate that NF- $\kappa$ B is not required for the fasl gene expression.<sup>58,59</sup> Nevertheless, pro-apoptotic or anti-apoptotic effects of NF- $\kappa$ B might depend on the cellular context in combination with a bewildering variety of activators.

Further support for the anti-apoptotic effect of NF- $\kappa$ B comes from observations in which NF- $\kappa$ B can protect cortical neurons from  $\beta$ -amyloid peptide-induced apoptosis in Alzheimer's disease.<sup>60,61</sup> Exposure of cortical neurons to  $\beta$ -amyloid peptide increased levels of I $\kappa$ B- $\alpha$  mRNA and protein and a consequent decrease in NF- $\kappa$ B activity.<sup>60</sup> Elevation of NF- $\kappa$ B activity by pretreatment of these cells with an antisense oligonucleotide to I $\kappa$ B- $\alpha$  protected them from  $\beta$ -amyloid peptide-induced apoptosis. Conversely, blockade of NF- $\kappa$ B activity by  $\kappa$ B decoy DNA was associated with enhanced  $\beta$ -amyloid peptide-induced mitochondrial dysfunction and cell apoptosis.<sup>61</sup> Moreover, data from the studies of rodent models of stroke or cardiac arrest suggested that NF- $\kappa$ B might possibly prevent ischemic neuronal degeneration.<sup>62</sup> The protective role of NF- $\kappa$ B on neurons was attributed to NF- $\kappa$ B-mediated transcription of genes encoding Bcl-2, Mn-SOD, and proteins regulating cellular calcium homeostasis.<sup>63</sup>

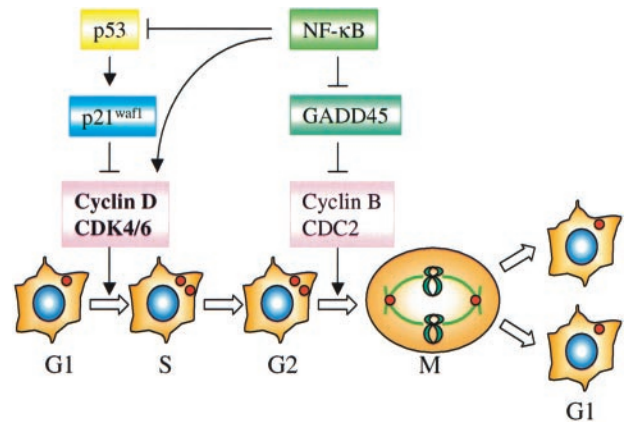
The vast majority of studies focused on the regulatory roles of NF- $\kappa$ B on apoptosis suggest that NF- $\kappa$ B is acting on the upstream pathways of apoptosis, either negatively or positively. Conversely, a few recent studies have demonstrated the possible regulation of apoptotic molecules on NF- $\kappa$ B. Of potential interest regarding regulation of NF- $\kappa$ B by apoptotic molecules are the observations of cross-competition between NF- $\kappa$ B and p53, a major pro-apoptotic protein.<sup>64–69</sup> The molecular events identified thus far as mediators of cross-competition between NF- $\kappa$ B and p53 can be roughly classified into two categories: the upstream kinases or other regulatory molecules that relay input signals into NF- $\kappa$ B and p53, and co-factors that affect transcriptional activities of NF- $\kappa$ B and p53. An earlier study conducted by Jung and colleagues<sup>70</sup> indicated that ATM, a major kinase responsible for DNA damage-induced N-terminal phosphorylation of p53, was involved in I $\kappa$ B- $\alpha$  phosphorylation in SV40 large T-transformed fibroblasts in response to ionizing radiation. In nontransformed fibroblasts, however, Ashburner and colleagues<sup>71</sup> demonstrated a lack of involvement of ATM in I $\kappa$ B- $\alpha$  phosphorylation. In an *in vitro* study, Liu and colleagues<sup>72</sup> reported that DNA-dependent protein kinase (DNA-PK), a kinase phosphorylating p53 in response to DNA damage, was able to phosphorylate the carboxyl terminus of I $\kappa$ B- $\alpha$  protein. On the functional level, the first evidence of mutual functional regulation



between NF- $\kappa$ B and p53 was from the observation that p53 could antagonize NF- $\kappa$ B activity by cross-competition for a limiting pool of the co-activator, p300.<sup>65</sup> In contrast, two recent studies indicated that p53 might activate NF- $\kappa$ B through an unknown mechanism<sup>73</sup> or stimulate the activity of NF- $\kappa$ B through induction of its target gene, p21<sup>Waf1</sup>, which inhibits cyclin E/Cdk2 activity and blocks its ability to compete with NF- $\kappa$ B for co-factors, such as p300 and CBP.<sup>74</sup>

Further evidence indicating that apoptotic molecules regulate NF- $\kappa$ B comes from study of caspase cleavage of NF- $\kappa$ B p65 subunit or I $\kappa$ B- $\alpha$  protein.<sup>75-77</sup> The cleavage of p65 by caspase-3 leads to a loss of the carboxyl-terminal transactivation domain.<sup>75</sup> The carboxyl terminal truncated p65 is transcriptionally inactive. The cleavage of I $\kappa$ B- $\alpha$  by caspase-3 has been observed in  $\gamma$ -radiation-induced apoptosis and NF- $\kappa$ B inhibition-induced apoptosis.<sup>77,78</sup> A caspase-3 cleavage site has been identified in the region of amino acids 26 to 32 of human I $\kappa$ B- $\alpha$  and 32 to 37 of chicken I $\kappa$ B- $\alpha$  protein.<sup>76</sup> The cleavage site of caspase-3 on I $\kappa$ B- $\alpha$  with the amino acid sequence D-R-H-D-S resembles the consensus caspase-3 cleavage site, D-X-X-D-G/S/A, where X represents any amino acid residue. Cleavage of I $\kappa$ B- $\alpha$  by caspase-3 creates a N-terminal truncated I $\kappa$ B- $\alpha$  protein that is resistant to degradation by proteasome in response to inducers of NF- $\kappa$ B, but is able to bind to and suppress NF- $\kappa$ B. Therefore, the role of caspase-3 cleavage on I $\kappa$ B- $\alpha$  and p65 is to ensure that the anti-apoptotic gene is suppressed and apoptotic process is not interrupted once the cells are committed to apoptotic elimination.

In contrast, several studies indicate that caspases might also participate in the activation of NF- $\kappa$ B under certain circumstances. One example supporting this notion is the involvement of *Dredd*, a caspase encoded by *Drosophila* *dredd* gene, in the endoprotease cleavage of the *Relish* protein.<sup>79</sup> Structurally similar to human p100 and p105, two precursor proteins of NF- $\kappa$ B family, *Relish* contains a N-terminal Rel homology domain and a C-terminal I $\kappa$ B-like region. On lipopolysaccharide stimulation, *Relish* undergoes a rapid cleavage between the Rel homology domain and I $\kappa$ B-like region. Proteasome inhibitors failed to prevent the cleavage of *Relish*. In contrast, introducing a dominant-negative mutant of *Dredd* to inhibit the caspase activity of *Dredd* significantly blocked the cleavage of *Relish*, indicating caspase, rather than proteasome, is required for the activation of NF- $\kappa$ B-like protein in *Drosophila*. In addition, in mammalian cells, caspase-8, caspase-10, and MRIT, three death effector domain-containing proteins, have been shown to be able to activate upstream signals, such as NIK and IKK, leading to the activation of NF- $\kappa$ B.<sup>80</sup> This activity seems to be mainly dependent on the interaction between the pro-domain of caspases and IKK. Furthermore, several recent studies suggested that other apoptosis-inducing proteins, such as Nod2, an Apaf-1 family member, and *Bcl10* and *vCLAP*, two caspase-recruitment domain-containing proteins, could also activate NF- $\kappa$ B through the interaction with IKK- $\gamma$  subunit of IKK complex.<sup>81-83</sup> However, it is hard to reconcile these observations with the notions



**Figure 3.** Involvement of NF- $\kappa$ B in cell-cycle regulation. NF- $\kappa$ B may facilitate cell-cycle transition from G<sub>1</sub> to S phase by antagonizing the activation or function of p53 and up-regulating cyclin D1 gene expression. NF- $\kappa$ B may also promote G<sub>2</sub>-to M-phase transition by down-regulating the expression of GADD45, a G<sub>2</sub>/M phase blocker that inhibits CDC2/cyclin B complex. →, activation; ⊥, inhibition.

that NF- $\kappa$ B is repressed in the cells undergoing apoptosis.<sup>33,77,78</sup>

### NF- $\kappa$ B and Cell-Cycle Regulation

It has been known for decades that multiple signals are required to maintain proper cell growth and tissue homeostasis.<sup>84</sup> Most cells within a normal tissue may be forced out of the active cell cycling into a quiescent (G<sub>0</sub>) state from which they may re-enter cell cycling under some future circumstances. In a mature tissue, cells may be induced to terminal differentiation by relinquishing their proliferative or cell-cycling potential. Control of the orderly progression of dividing cells through the G<sub>1</sub>, S, G<sub>2</sub>, and M phases of the cell cycle in eukaryotic cells relies on a series of cell-cycle regulatory proteins, mainly cyclins that exert their function by binding to and activating a number of specific cyclin-dependent kinases (CDKs). The CDK activity is further modulated by kinases and phosphatases that phosphorylate and dephosphorylate CDK, respectively. Moreover, CDKs are subject to regulation by association with one of a number of specific CDK inhibitors or cell-cycle checkpoint proteins, such as p21<sup>Waf1</sup>, p16<sup>INK4a</sup>, p27<sup>Kip1</sup>,<sup>85</sup> and growth arrest and DNA-damage protein 45 (GADD45) (Figure 3).<sup>86</sup>

Overwhelming evidence during recent years demonstrates that a variety of stress inducers, including DNA-damaging agents, activate checkpoint function of cells, leading to a cell-cycle arrest. Several checkpoints exist in the G<sub>1</sub>/S phase, G<sub>2</sub> phase, and M phase of cell cycle. In mammalian cells, the control of the S-phase checkpoint requires the p53 tumor suppressor protein that governs the expression of CDK inhibitor, p21<sup>Waf1</sup>.<sup>87,88</sup> The activation of the G<sub>2</sub>/M phase checkpoint is dependent on the phosphorylation-dependent inactivation of CDC25C phosphatase by checkpoint kinases 1 or 2 (Chk1 or Chk2) and the induction of GADD45, an inhibitor for the G<sub>2</sub>/M phase cyclin B/CDC2 complex.<sup>89,90</sup> An additional checkpoint, the spindle checkpoint, has been identified

in a later stage of M phase.<sup>84,85</sup> This checkpoint arrests mitotic progression if the spindle is not properly assembled, or if the chromosomes are not correctly oriented and attached to the spindle. All of the checkpoints are essential for maintaining genomic stability by allowing cells to have enough time to repair damage, thus, protecting the organism from the deleterious consequences of mutation.

The relationship between NF- $\kappa$ B and apoptosis has been intensively explored during the last few years, whereas only limited information is available regarding the possible involvement of NF- $\kappa$ B in cell-cycle regulation in cellular response to a variety of stress signals. A critical role for NF- $\kappa$ B in cell-cycle progression was suggested by earlier observations that NF- $\kappa$ B activity was elevated during the G<sub>0</sub> to G<sub>1</sub> cell-cycle transition in mouse fibroblasts.<sup>91</sup> A series of recent studies has begun to elucidate that in addition to fibroblasts, NF- $\kappa$ B activation was required for cell cycling in other types of cells, such as regenerating liver cells and estrogen receptor-negative breast cancer cells.<sup>91-95</sup> It was also found that the levels of NF- $\kappa$ B activation were linked to signaling that controls cell-cycle progression in HeLa cells and Jurkat T cells.<sup>74,96</sup> Inhibition of NF- $\kappa$ B caused impairment of cell-cycle progression in human glioma cells<sup>97</sup> and a retarded G<sub>1</sub>/S transition in HeLa cells.<sup>98</sup> The identification of NF- $\kappa$ B binding sites in the promoter region of cyclin D1 gene provided direct evidence for the contributions of NF- $\kappa$ B to the cell cycle.<sup>50-52,95</sup> Cyclin D1, in association with cyclin-dependent kinases, CDK4 and CDK6, promotes G<sub>1</sub>/S phase transition through CDK-dependent phosphorylation of pRb, thereby releasing the transcription factor E2F, which is required for the activation of S phase-specific genes.<sup>99-101</sup> Two NF- $\kappa$ B binding sites in the human cyclin D1 promoter have been identified. Inhibition of NF- $\kappa$ B by a degradation-resistant I $\kappa$ B- $\alpha$  caused a pronounced reduction of serum-induced cyclin D1 expression accompanied by a decrease of cyclin D1-associated kinase activity and delayed phosphorylation of pRb.

In contrast, several recent reports also indicated that NF- $\kappa$ B activation is necessary to cause cell-cycle arrest and/or induce cells to commit to terminal differentiation. Overexpression of NF- $\kappa$ B p65 or c-Rel arrests G<sub>1</sub>/S cell-cycle transition in pro-B cells and HeLa cells, respectively.<sup>96,102</sup> In HeLa cells, overexpression of c-Rel arrests cells at the G<sub>1</sub>/S phase because of the stabilization of p53 protein, which can subsequently activate the expression of p21<sup>Waf1</sup>, a potent inhibitor of CDK2.<sup>96</sup> The elevated levels of p21<sup>Waf1</sup> correlated with the accumulation of the hypophosphorylated form of pRb and a decrease in E2F DNA binding. It is unclear how overexpression of c-Rel resulted in a prolonged half-life of p53 protein. In pro-B cells, although overexpression of c-Rel exhibited no effect on cell-cycle regulation, overexpression of p65 caused G<sub>1</sub> arrest and subsequent apoptosis. This G<sub>1</sub>-arresting effect of p65, however, seems to be dependent on cell developmental stage, because overexpression of p65 did not cause G<sub>1</sub> arrest in mature B cells. It remains unsettled whether manipulation of NF- $\kappa$ B signaling using protein overexpression can lead to consequences that

are physiologically relevant. If it is, one may speculate that inhibition of NF- $\kappa$ B should cause over-cycling or hyperproliferation. Indeed, data obtained by gene inactivation of IKK- $\alpha$  in the mouse indicated an unexpected excessive proliferation of the skin basal layer because of the absence of epidermal differentiation.<sup>103,104</sup> NF- $\kappa$ B activity could not be found in keratinocytes from IKK- $\alpha$ -null mouse skin. These results point to a unique role for NF- $\kappa$ B in the epidermis, that is, NF- $\kappa$ B forces keratinocytes out of cell cycle and subsequent terminal differentiation. In this regard, the response of the epidermal keratinocytes to NF- $\kappa$ B seem to be opposite from that of other cell types, such as lymphocytes and macrophages, where NF- $\kappa$ B seems to promote cell-cycle transition.<sup>50-52,95</sup>

Although most of the studies so far addressed the effects of NF- $\kappa$ B on G<sub>1</sub>/S phase regulation, the question of whether NF- $\kappa$ B also contributes to G<sub>2</sub>/M phase transition has not been explored. In a recent study in human bronchial epithelial cell line, BEAS-2B, we found that NF- $\kappa$ B inhibition by stable expression of a kinase mutated form of IKK- $\beta$  potentiated toxic metal-induced G<sub>2</sub>/M cell-cycle arrest.<sup>105</sup> First, flow cytometric analysis demonstrated that at 48 hours after arsenite treatment, BEAS-2B cells expressing a kinase-mutated form of IKK- $\beta$  showed a marked dose-dependent increase of cells arrested in the G<sub>2</sub>/M phase and a corresponding decrease in the number of cells in G<sub>1</sub> phase. Second, a dose-dependent induction of GADD45 protein was observed in cells treated with arsenite. This induction of GADD45 by arsenite seems to be dependent on the activation of c-Jun-N-terminal kinase, because blockage of c-Jun-N-terminal kinase activation by expression of a dominant-negative SEK1 vector decreased the induction of GADD45. On the other hand, inhibition of NF- $\kappa$ B by expressing a kinase-mutated form of IKK- $\beta$  increased GADD45 induction by arsenite. Third, analysis for the expression of CDC25 family members revealed that arsenite induced *de novo* CDC25A expression, but markedly reduced the levels of CDC25B and CDC25C proteins, two phosphatases dephosphorylating and activating CDC2/cyclin B complex required for the transition of the cell cycle from G<sub>2</sub> to M phase. The effects of Cr(VI) on the regulation of the cell cycle were also determined and revealed to be more complicated. Although Cr(VI) was able to induce GADD45 and suppress both CDC25B and CDC25C, it had no effect on CDC25A. Cell-cycle-profiling studies showed that whereas a lower concentration of Cr(VI) (0.25  $\mu$ g/ml) promoted cell-cycle transition, higher concentrations of Cr(VI) (1 to 4  $\mu$ g/ml) arrested cells at S phase. In the case of vanadate-induced cell-cycle regulation, the cell-cycle-arresting effect of vanadate seems to be dependent on the status of NF- $\kappa$ B activation. In normal epithelial cells, vanadate exhibited less effect on cell-cycle transition. However, in the cells where NF- $\kappa$ B activation was specifically inhibited, vanadate showed a marked G<sub>2</sub>/M phase-arresting effect. However, vanadate was unable to induce the expression of GADD45, an inhibitor of cyclin B/CDC2 complex required for G<sub>2</sub>/M transition (Chen et al, unpublished observations).

## NF- $\kappa$ B and Oncogenesis

The ability of NF- $\kappa$ B to suppress apoptosis and to regulate cell-cycle transition clearly indicates that NF- $\kappa$ B may participate in many aspects of oncogenesis. Indeed, elevation of NF- $\kappa$ B activity is evident in a number of human cancers, including breast cancer,<sup>106</sup> non-small cell lung carcinoma,<sup>107</sup> thyroid cancer,<sup>108</sup> T- or B- lymphocyte leukemia,<sup>109</sup> melanoma,<sup>110</sup> colon cancer,<sup>111</sup> bladder cancer,<sup>112</sup> and several virally induced tumors.<sup>113–115</sup> The earliest evidence for a role for NF- $\kappa$ B in oncogenic transformation has been derived from the fact that v-Rel, a highly oncogenic retroviral homologue of c-Rel, causes carcinogenesis in avian lymphoid cells.<sup>108</sup> Later studies suggested that v-Rel also has the capacity of transforming mammalian cells *in vivo*.<sup>116</sup> Transgenic mice expressing v-Rel under the control of the T-cell-specific *lck* promoter develop T-cell lymphomas. Inhibition of NF- $\kappa$ B by overexpression of a degradation-resistant I $\kappa$ B- $\alpha$  delays the development of T-cell lymphomas and prolongs the survival of v-Rel transgenic mice.<sup>116</sup>

Chromosomal alterations of NF- $\kappa$ B family genes provided additional evidence for the role of NF- $\kappa$ B in oncogenesis. It has been demonstrated that genes encoding c-Rel, NF- $\kappa$ B2 (p100/p52), p65/RelA, and Bcl-3 proteins are all located within breakpoint regions of the genome that are involved in oncogenic rearrangements or amplifications. Rearrangement of *nfk2b2* gene by t(10,14) chromosomal translocation causes deletions of sequences encoding the ankyrin repeat motif of p100. Consequently, this carboxyl terminal truncated p100 is constitutively located in the nucleus of cells, which has been originally found in a case of B-cell non-Hodgkin's lymphoma and observed in a number of lymphoid neoplasms, particularly cutaneous lymphomas.<sup>117–119</sup> Rearrangement and amplification of c-Rel gene has also been found in numerous non-Hodgkin's lymphomas and cancer cell lines.<sup>108,120</sup> The *bcl-3* gene, which encodes an I $\kappa$ B-like protein that regulates transcriptional activity of NF- $\kappa$ B p50 or p52 homodimer, was identified as a [t(14, 19)(q32; q13.1)] chromosomal translocation in many cases of chronic lymphocytic leukemia.<sup>121</sup> Unlike rearrangement of the *nfk2b2* gene, alterations at the *bcl-3* locus do not truncate or change the coding sequence, but rather cause overexpression of *bcl-3* mRNA. In malignant Hodgkin and Reed-Sternberg (H/RS) cells from Hodgkin's lymphoma, mutations in the I $\kappa$ B- $\alpha$  gene have been detected and are suggested to cause a sustained activation of NF- $\kappa$ B.<sup>122</sup>

Accumulating evidence reveals that tumorigenesis or oncogenesis is a multistep process and that these steps reflect defections in regulatory circuits that govern normal cell proliferation, differentiation, and death.<sup>123</sup> Although abnormal activation or function of NF- $\kappa$ B has been clearly demonstrated in the initiation or facilitation of oncogenesis, the central question that has to be answered is: how many and what steps are influenced by NF- $\kappa$ B. NF- $\kappa$ B has been shown to antagonize the function of p53 as discussed earlier.<sup>65</sup> Obviously, this antagonism of p53 by NF- $\kappa$ B will result in the evasion of cells from stress-induced cell-cycle arrest and/or programmed cell death

and consequently sensitize the cells for genomic instability. Furthermore, NF- $\kappa$ B could promote cell-cycle transition by a direct transcriptional up-regulation of the cyclin D1 gene.<sup>50–52,95</sup> Although it remains to be confirmed, this increased expression of cyclin D1 may possibly provide cells with an uncontrolled or limitless replicative potential. Up-regulation of anti-apoptotic genes, such as cIAP1, cIAP2, XIAP, and bcl-xl, by NF- $\kappa$ B,<sup>4</sup> is an additional mean of cells to escape from or resist signal-induced apoptosis. Other NF- $\kappa$ B-regulated genes include those encoding intercellular adhesion molecule-1,<sup>5</sup> extracellular matrix protein tenascin-C,<sup>124</sup> vascular endothelial growth factor,<sup>124</sup> chemokines, and cyclooxygenase-2.<sup>124</sup> These gene products are directly associated with the tumor cell metastasis and tumor tissue angiogenesis.

The key role that NF- $\kappa$ B plays on multiple steps of oncogenesis makes this factor a central and favorable target for therapeutic intervention of cancer, especially, certain types of leukemia or lymphomas.<sup>125</sup> Indeed, experimental data suggest that inhibition of NF- $\kappa$ B by antisense oligonucleotides to *relA*, degradation resistant I $\kappa$ B- $\alpha$ , and aspirin or nonsteroidal anti-inflammatory drugs, could enhance the efficacy of cancer chemotherapies and radiation.<sup>126,127</sup> Studies by Wang and co-workers<sup>55,128</sup> showed that inhibition of NF- $\kappa$ B by infecting the cells with an adenovirus carrying a modified form of I $\kappa$ B- $\alpha$  (superrepressor I $\kappa$ B- $\alpha$ ) leads to dramatically enhanced apoptosis of HT1080 fibrosarcoma cells in response to ionizing radiation or daunorubicin treatment. Consistent with these reports, working with pancreatic cancer cell lines exposed to VP16 or doxorubicin, Arlt and colleagues<sup>129</sup> demonstrated recently that NF- $\kappa$ B inhibition by pharmacological proteasome inhibitors or transfection of the cells with a N-terminal-truncated I $\kappa$ B- $\alpha$  variant efficiently reduces chemoresistance of these cells. Using similar or different approaches to inhibit NF- $\kappa$ B, this effect has also been noted in a variety of other cell types including non-small cell lung cancers,<sup>130</sup> head and neck squamous carcinomas,<sup>131</sup> human myeloblastic leukemia cells,<sup>132</sup> colorectal cancer,<sup>133</sup> and bladder cancer cells.<sup>112</sup> Despite these encouraging observations, however, care has to be taken when using different approaches to inhibit NF- $\kappa$ B that might be attributable to the process of oncogenesis. Indeed, different approaches for the inhibition of NF- $\kappa$ B does not necessarily lead to the same extents of inactivation of NF- $\kappa$ B because of the existence of functionally and stoichiometrically different NF- $\kappa$ B complexes that respond to different activation signals.<sup>5</sup> Also, the inhibitory effect of NF- $\kappa$ B inhibitors can vary considerably between different cell types because of unique simultaneous or asynchronous events triggered by these inhibitors in any given cell type.<sup>134</sup>

## Summary

The detailed molecular mechanisms by which the NF- $\kappa$ B transcription factor contributes to cell growth control, such as cell apoptosis, cell-cycle transition, and oncogenesis remain to be further determined. One of the major challenges in understanding mechanisms of cell



growth regulation by NF- $\kappa$ B in response to environmental stress is to elucidate how signal transduction pathways are activated and how signaling cross-talk and specificity are achieved when several signaling pathways are activated simultaneously by stress inducers that elicit different cellular responses. For instance, why does activation of the NF- $\kappa$ B, an anti-apoptotic transcription factor, coincide with obvious apoptotic features in cells undergoing stress responses? Because many stress inducers and their mediators are highly reactive but nonspecific, an activation of only one specific signaling pathway is hard to achieve in the cells in response to a particular inducer. Even in a single signaling pathway, because of their highly reactive and nonspecific characteristics, certain stress inducers and their mediators can in principle induce conflicting signals by affecting signaling molecules at different levels. A good example is the effects of oxidative stress on NF- $\kappa$ B signaling pathway. It has been frequently observed in certain types of cells that oxidative stress amplified or potentiated NF- $\kappa$ B activation, whereas at the same time oxidation of IKK or NF- $\kappa$ B proteins inhibited NF- $\kappa$ B function. Translating the knowledge gained by studying the connections among NF- $\kappa$ B activation, cell apoptosis, cell-cycle regulation, and oncogenesis may aid in identifying novel preventive and therapeutic measures for diseases, such as chronic inflammation and cancer.

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