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COMMENTARY

Innovative Treatment Programs against Cancer

II. NUCLEAR FACTOR-κB (NF-κB) AS A MOLECULAR TARGET

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ABSTRACT. Nuclear factor-κB (NF-κB) activity affects cell survival and determines the sensitivity of cancer cells to cytotoxic agents as well as to ionizing radiation. Preventing the protective function of NF-κB may result in chemo- and radio-sensitization of cancer cells. Therefore, NF-κB has emerged as one of the most promising molecular targets in rational drug design efforts of translational cancer research programs. BIOCHEM PHARMACOL **57**;1:9–17, 1999. © 1998 Elsevier Science Inc.

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Activation of the NF-κB†/Rel family of transcriptional factors regulates the expression of genes that participate in pathways involving inflammation, cell proliferation, and susceptibility to apoptosis [1–6]. The various transcriptional subunit members of the Rel family exist as inactive hetero- or homodimers in the cytoplasm of unstimulated cells, the prototype NF-κB being composed by dimerization of the proteins for p65 (RelA) and either p50 or p105 (NF-κB1), which, when unprocessed, contains both the p50 sequence and an inhibitory C-terminal IκB-like sequence that is proteolytically degradable [2–6]. For a pictorial illustration of the signalling pathway leading to NF-κB transcriptional activity, see Fig. 1; Table 1 shows the relationship between Rel family members and IκB family members.

Activation of the transcriptional capability of factors such as NF-κB occurs following a stimulus provoking an intracellular oxidative response, for example, receptor binding of proinflammatory cytokines such as IL-1 or TNF-α, or exposure to other inducers of ROI [1–7]. Oxidative stress activates upstream tyrosine and serine/threonine kinases that phosphorylate, either directly or as a consequence of a cascade, the inhibitory IκB subunits, which retain Rel-family dimers in the cytoplasm, thereby allowing translocation of the transcriptional factors to the nucleus where they can bind to specific gene promoter sites [1–7].

The pathway mediated by proximal activation of MAP family serine/threonine kinases confers NF-κB activation by sequential phosphorylations that cause ubiquitination and proteasomal degradation of IκB proteins, in addition to phosphorylating sites on Rel proteins that facilitate their nuclear localization [1–7]. The signalling pathway that is reliant on serine/threonine kinases appears to involve members of the MAPK family. By correlating several reports that describe activation of NF-κB in response to stimuli such as TNF-α, IL-1, LPS, insulin, PMA, or a number of other factors [1, 7–9], a likely signalling sequence emerges. A general description is that stimulation of cells by these agents activates an MAPKK that phosphorylates and activates an MAPK that subsequently activates another kinase, which, in turn, activates the kinase(s) directly responsible for phosphorylating the IκB α, β, and ϵ subunits. The serine-phosphorylated IκB subunits then become ubiquitinated and are destined for proteasomal degradation, thereby freeing the NF-κB dimer subunits for translocation to the nucleus where they can promote gene transcription.

More specifically, the MAPKK that copurified with IκB kinase activity was MEKK-1, and this MAPKK was tightly associated with the MKP-1 MAPK phosphatase [1]. The identity of the downstream MAPK is uncertain; reports by several groups have designated it as NF-κB-inducing kinase (NIK) [7], $p90^{rsk1}$ [9–11], MNK1 [11], or ERK [11]. Two recent reports make clear that two kinases directly responsible for serine phosphorylation of the IκB subunits are IKK-α (or IKK-1) and IKK-β (or IKK-2) [7, 12]. These kinases appear to associate as components of a "signalsome" responsible for phosphorylation of the NF-κB inhibitory complex subunits, IκB-α, -β, and -ϵ. Phosphorylation of these inhibitory proteins on two serine residues destines

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† *Abbreviations:* NF-κB, nuclear factor-κB; IL, interleukin; TNF, tumor necrosis factor; ROI, reactive oxygen intermediates; PDTC, pyrrolidinedithiocarbamate; HVS, herpesvirus saimiri; TGF, transforming growth factor; IGF, insulin-like growth factor(s); IRS-1, insulin receptor substrate 1; CCK, cholecystokinin; ALAD, δ-aminolevulinic acid dehydratase; IR, ionizing radiation; LPS, lipopolysaccharide; PMA, 12-phorbol 13-myristate acetate; PTK, protein tyrosine kinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; HTLV, human T lymphotropic virus; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; P13-kinase, phosphatidylinositol 3′-kinase; and PKC, protein kinase C.

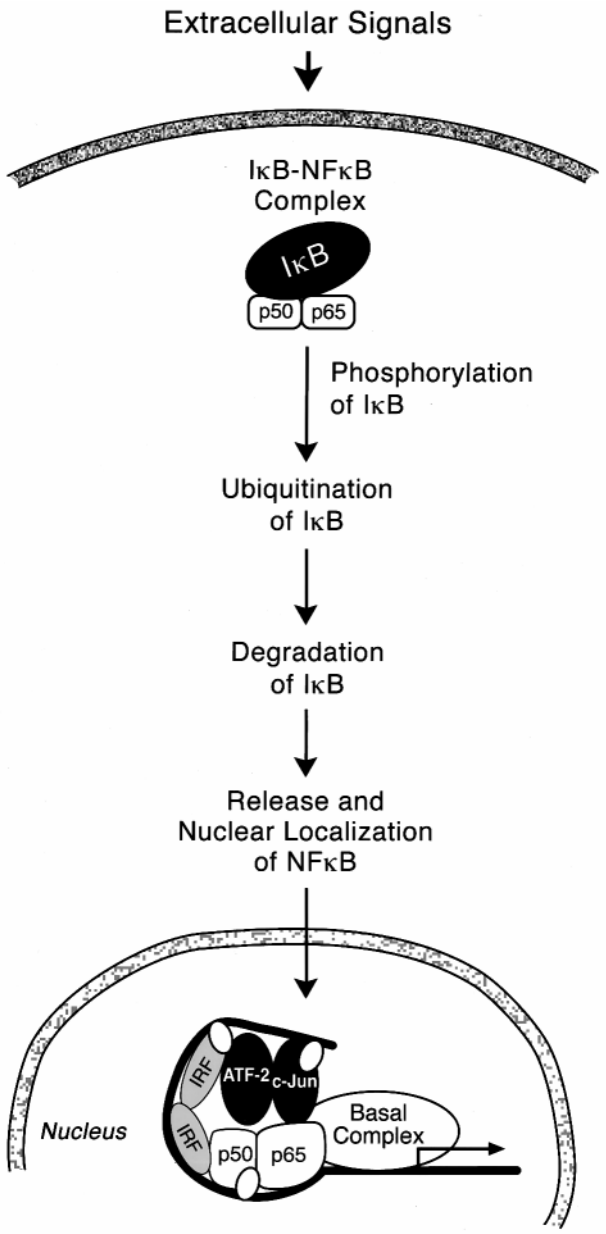

FIG. 1. A number of extracellular stimuli that activate signal transducers of the NF-κB family via up-regulating the activity of membrane-proximal kinase(s). This causes downstream phosphorylation of inhibitory components of the NF-κB complex in the cytosol that eventuates their proteolytic degradation. After activation, the NF-κB heterodimer gains access to the nucleus and synergizes with other transcriptional activators to form a complex that promotes gene transcription. The example shown is the IFN-β gene promoter, but other binding sites and complex formations are used for up-regulating the transcription of genes fundamental to the decision between cell cycle progression or apoptosis, such as *c-myc*. Abbreviation not defined in the text: IRF, interferon-regulatory factor.

them for conjugation to ubiquitin multimers that, in turn, target the inhibitors for proteasomal degradation.

A couple of reports [13, 14] describe an alternative pathway of NF-κB activation that is dependent on tyrosine phosphorylation, rather than on serine phosphorylation.

TABLE 1. Relationship between Rel family members and IκB family members

Proteins	Rel homology domain	Ankyrin repeats
p65 (RelA)	+	−
c-Rel	+	−
RelB	+	−
Dorsal	+	−
Dif	+	−
p105/p50 (NF-κB1)	+	+
p100/p52 (NF-κB2)	+	+
IκB-α	−	+
IκB-β	−	+
Bcl-3	−	+
Cactus	−	+

The Rel domain is approximately 300 amino acids in length and is required for dimerization of all NF-κB-related family members. The Rel domain also controls interaction with IκB family members and contains a signalling portion that facilitates nuclear localization. Ankyrin repeats are found in all IκB family members and are required for binding of IκB proteins to the Rel family members. The Dorsal, Dif, and cactus proteins are found in *Drosophila*, and the others are found in mammalian cells.

The pathway involving tyrosine phosphorylation of IκB proteins appears more direct, in that the Src-family $p56^{lck}$ kinase is proximally activated (for instance, by IR) and tyrosine phosphorylates the IκB-α subunit; this allows for NF-κB activation in the absence of IκB degradation [13].

Intracellular ROI generated by exposure of B-lymphoid cells to IR or H_2O_2 resulted in activation of NF-κB that was dependent upon up-regulation of PTK activity [14]. The PTK inhibitor herbimycin A and the radical scavenger *N*-acetyl-L-cysteine were each able to prevent the ROI-induced activation of NF-κB. When H_2O_2 exposure was combined with inhibition of protein tyrosine phosphatase activity using vanadate, activation of the Src-family $p56^{lck}$ and $p59^{fyn}$ tyrosine kinases was apparent. Whereas this might be interpreted as a proximal portion of the kinase cascade leading to downstream activation of serine/threonine kinases, the other study [13] shows that treatment of T-lineage cells with pervanadate, which is equivalent to vanadate in terms of protein tyrosine phosphatase inhibition and PTK activation, led to NF-κB activation through tyrosine phosphorylation of IκB-α. The pervanadate-induced IκB-α phosphorylation and NF-κB activation required the presence of $p56^{lck}$. Notably, the activation of NF-κB via tyrosine phosphorylation of IκB-α did not require proteasomal degradation of the IκB-α protein. These PTK-dependent responses may not be identical because oxidant-dependent pathways, such as those involving IR and H_2O_2, invoke NF-κB activation that can be prevented using PDTC, and inhibition of phosphatase activity does not depend upon ROI generation, since it is not inhibitable by PDTC [15]. By whichever manner PTK activation stimulates NF-κB activity, either indirectly through upstream interaction with MAP family kinases or by direct phosphorylation of IκB subunits, modulation of NF-κB transcriptional capacity through changes in PTK activity may have an effect upon the susceptibility of malignant cells to therapeutic modalities.

A mutant pre-B-cell line failed to activate NF-κB in response to a number of stimuli, including stimulation by LPS, PMA, Taxol, or IL-1 [15]. This is the result of an inability to phosphorylate and degrade the IκB-α, -β, and -ϵ inhibitory subunits, even though no mutations were found in IκB or NF-κB subunits. The defective portion of the response pathway appears to be mediated by intracellular ROI induced by receptor binding because the PDTC antioxidant was able to duplicate the same stimuli. In contrast, PDTC-insensitive stimuli, such as hyperosmotic shock, HTLV-1-derived Tax *trans*-activator, or phosphatase inhibitors, bypassed the defective portion of the pathway and caused IκB subunit degradation and NF-κB activation [15]. This study shows that several stimuli that utilize ROI in their signal transduction operate through a common messenger involved in triggering degradation of the α, β, and ϵ subunits of the IκB complex.

As discussed below, treatment of B lymphoma cells with the TPCK protease inhibitor resulted in apoptosis by preventing IκB degradation and NF-κB activation in otherwise unstimulated cells [16]. In addition, treatment of these cells with the PDTC antioxidant also prevents NF-κB activation by inhibiting *de novo* phosphorylation of IκB [16, 17]. Seemingly because a basal level of oxidant-induced IκB degradation is necessary for maintenance levels of NF-κB/Rel activity, PDTC treatment causes the cells to die by apoptosis.

This report discusses the protective function of NF-κB in cells exposed to cytotoxic agents or oxidative stress and outlines many means by which the activation or function of NF-κB family members can be prevented, with the aim of conferring enhanced efficacy on existent and novel therapeutic modalities for cancer treatment.

NF-κB AND CELL SURVIVAL

Because activation of NF-κB and related factors in response to oxidative stress is a major protective mechanism for the cell, due to the transcriptional activation of particular genes, inhibition of such a response would serve to sensitize cells to apoptotic cell death resulting from exposure to such stimuli. As an example, T-lineage lymphoid cells treated with TNF-α invoke an intracellular signalling response via two pathways. One pathway, if unopposed, would upregulate the apoptotic program and kill the cell. However, since receptor binding of TNF-α also activates the NF-κB response, TNF-α is more commonly a growth factor for T-cells, rather than a "death factor." Nonetheless, if activation of NF-κB is prevented, binding of TNF-α to its receptor is lethal [18]. The gene encoding human c-IAP2 requires NF-κB for induction by TNF-α. When overexpressed in mammalian cells, the c-IAP2 protein activates NF-κB and suppresses TNF-mediated cytotoxicity [18]. Both of these c-IAP2 activities can be blocked *in vivo* by co-expressing a dominant form of IκB that is resistant to TNF-induced degradation. Unlike wild-type c-IAP2, a mutant form of c-IAP2 that lacks the C-terminal RING domain inhibits NF-κB induction by TNF-α and enhances resultant apoptotic cell death [18]. Transcription of the gene for the c-IAP2 protein appears to be a critical component of a positive feedback loop involving NF-κB that spares TNF-mediated death via an IκB targeting mechanism. Similar sensitization of cells exposed to IR or chemotherapeutic drugs that invoke ROI production results from interference in the Rel-family response mechanism [19, 20].

Treatment of immature B-lymphoma cells with an antibody against their surface IgM causes an early, transient increase in Rel-family DNA binding that induces a short-term rise in c-*myc* transcription [21, 22]. The anti-IgM-treated cells subsequently demonstrate a sharp drop in transcription and c-*myc* expression to below pretreatment baseline levels that appeared to result from overexpression and binding of p50 homodimers rather than p50/p65 heterodimers [22]. Thus, the apoptosis that follows IgM ligation in these cells results from reduced levels of c-*myc* gene transcription, and this is mediated by skewing the ratio of Rel-family subunit representation, activation, and DNA binding. The response of the c-*myc* pathway to surface IgM ligation could be prevented by PDTC antioxidant treatment of the cells or overexpression of the IκB-α inhibitory subunit [22]. Moreover, a basal level of c-*myc* expression appears to be required for survival even in unstimulated cells since apoptosis of the B-lymphoma cells ensued following enhanced expression of Mad1, which has been shown to reduce levels of functional c-Myc [21]. The baseline activity of NF-κB seems to be important for maintaining a level of c-*myc* transcription required for survival. This was shown by the addition to unstimulated cells of TPCK, which blocks the normally rapid turnover of the IκB-α NF-κB/Rel inhibitor, causing a drop in Rel-related DNA binding and decreased c-*myc* expression that eventuated apoptotic cell death [21]. These observations suggest that a c-*myc*-specific inhibitor could be incorporated into malignant cell-focused therapeutic regimens.

VIRUSES AND NF-κB

NF-κB is critical for inducible expression of not only multiple cellular survival genes but also for expression of viral genes (e.g. HIV), and viruses have evolved a number of means for modulating NF-κB activity [23]. Activation of NF-κB is required for HIV gene transcription as well as viral replication and, in HIV-infected cells, the integrated proviral enhancer DNA sequence is permanently occupied by NF-κB p50/p65 heterodimers [24]. The HIV gp120 surface protein potentiates TNF-α-mediated activation of NF-κB by stimulating a signalling pathway involving $p56^{lck}$ PTK and increased formation of ROI such as H_2O_2 [25]. In Lck-deficient T-cells, gp120 is unable to generate H_2O_2 or to oxidize GSH, and had no effect on TNF-induced NF-κB activation. Therefore, Lck has an active role in increasing the oxidative state in cells to amplify TNF-mediated binding of NF-κB to cellular and viral DNA.

The NF-κB activation via gp120 can be inhibited by the antioxidant butylated hydroxyanisole [25]. In monocytic U937 cells, as early as 2 hr after adding the Z-LLL-H proteasome inhibitor, constitutive activation of NF-κB and HIV-1 genome expression were inhibited [24], presumably by preventing IκB degradation.

The avian virus v-*rel* gene is a member of the NF-κB family and causes aggressive immature T-cell malignancies in mice [26]. The transforming activity of v-Rel in transgenic mice results from DNA binding of v-Rel homodimers [26]. Infection by HVS is known to promote T-cell transformation *in vitro*, and the HVS-encoded Tip and Tap proteins modulate Lck activity [27]. Jurkat T-cells transfected with Tip and Tap had 40-fold higher NF-κB activity [27].

The genomes of human adenoviruses encode several regulatory proteins, two of which are the differentially spliced gene products E1A and E1B [28]. The E1A protein induces ROI, phosphorylation of IκB-α followed by its degradation, and activation of NF-κB. In contrast, the E1B protein prevents activation of NF-κB and its transcriptional activity [28]. The adenoviral E1B protein may be useful in suppressing the actions of NF-κB in therapeutic contexts.

MODULATION OF NF-κB FUNCTION AND NF-κB-LINKED SIGNAL TRANSDUCTION EVENTS

Each of the steps in the NF-κB-linked signalling cascade can be influenced, sometimes by several different means. Of particular interest, from the perspective of enhancing the efficacy of cancer cell eradication, is that there appear to be many ways to interfere with this signalling cascade in order to prevent NF-κB activation and/or function. These methods range from treatment of the target cells with a particular cytokine through inactivation of proteasome function, with specific inhibition of messenger molecules filling the gap between these two ends of the spectrum.

The ultimate focus for prevention of NF-κB activation is preservation of the IκB inhibitory proteins. The reports describing the IKK kinases confirmed the importance of this by showing that mutant versions of these proteins with defective catalytic activity prevented IL-1- and TNF-α-stimulated cells from activating NF-κB because of impaired proteolytic degradation of IκB subunits [1, 7]. Consistent with this, TPCK, a serine/threonine protease inhibitor that prevents degradation of the IκB-α subunit, suppresses activation of NF-κB and induces apoptosis in cells from B-cell lymphoma lines [16, 29]. In contrast, IKK mutants with constitutive IκB kinase activity induce NF-κB activation in the absence of receptor-mediated cell stimulation [1]. Modulation of the activation and function of the IKK signalsome using drugs, or by other means, could play a major role in improving the success of cancer therapy.

Glucocorticoids

Glucocorticoids, such as dexamethasone, are potent anti-inflammatory, immunosuppressive, and cytotoxic agents that are commonly used in chemotherapy for lymphoid neoplasms. These corticosteroids inhibit synthesis and expression of many cytokines and cell-surface molecules required for immune function and survival. A report [12] shows that glucocorticoids inhibit NF-κB activation both *in vivo* and *in vitro* and that this inhibition is conferred by induction of IκB-α protein expression, thereby trapping NF-κB in inactive cytoplasmic complexes. It may be that suppression of c-*myc* expression contributes to the death of lymphoid cells exposed to therapeutic doses of corticosteroids.

TGF-β1

The TGF-β1 cytokine is well-known for its role as an immunosuppressant that often antagonizes the stimulatory effect of TNF-α [30]. A recently published report [31] clarifies this activity by showing, in B-cell lymphoma line cells, that TGF-β1 treatment decreases c-*myc* expression and causes apoptosis. The reduction in c-Myc results from TGF-β1-binding provoking elevated IκB-α expression and, consequently, lower levels of NF-κB/Rel activation. Engagement of the CD40 co-receptor protected these cells from the TGF-β1-induced apoptosis by maintaining NF-κB activity and c-*myc* expression. Administration of TGF-β1 to patients with refractory lymphoid malignancies may sensitize the clonogenic cells to eradication through suppression of the NF-κB/c-*myc* pathway.

Exposure of cells expressing normal p53 induces synthesis of the cyclin-dependent kinase inhibitor $p21^{cip1}$ that contributes to cell-cycle delay, and the same effect can be induced by TGF-β1 in a p53-independent manner [32]. TGF-β1 treatment of human carcinoma cells enhanced the sensitivity to lethality conferred by subsequent radiation exposure. Moreover, sublethally irradiated carcinoma cells were significantly more sensitive to TGF-β1-induced apoptosis than unirradiated controls [32]. Their mutual effects on Cip1 expression may contribute to their cooperative effects upon induction of cell death.

Another study [33] showed that TGF-β1 enhances the lethal effect of DNA-damaging agents on human cancer cells through synergy with UV-C, γ-rays, cisplatin, methotrexate, and 5-fluorouracil. In this study, the sole effect of TGF-β1 when used alone was to inhibit proliferation without inducing apoptosis.

TGF-β1 has also been shown to reduce the activities of intracellular glutathione peroxidase and catalase [34]. This results in increased intracellular levels of peroxides, causing peroxidative damage to key proteins. Exposure of TGF-β1-treated cells to exogenous H_2O_2 caused enhanced apoptotic DNA cleavage. Augmented production of ROI by TGF-β1 through suppression of antioxidant enzymes, in combination with inhibition of the protective NF-κB response, may

amplify cellular damage and consequent apoptosis. Therefore, TGF-β1 may be a useful adjunct to chemotherapy.

IGF and IRS-1

IGF initiate tyrosine phosphorylation of IRS-1, and the gene for this protein is up-regulated in some malignancies [35]. When cancer cells that are sensitive to TGF-β1-induced apoptosis are forced to overexpress IRS-1, they become resistant to cell death promoted by TGF-β1 exposure. Overexpression of IRS-1 in NIH 3T3 cells causes malignant transformation with constitutive activation of the MAPK signalling cascade. IRS-1-mediated signalling may provide survival signals that protect against TGF-β1-induced apoptosis.

Gliotoxin

Gliotoxin (epipolythiodioxopiperazine) is a metabolite produced by *Aspergillis fumigatus* and other pathogenic fungi, which is selectively toxic at nanomolar concentrations to lymphocytes and macrophages [36–40]. Gliotoxin elicits potent immunosuppressive activity *in vivo* through induction of apoptosis in thymocytes, lymphocytes in spleen and lymph nodes, and selective apoptotic depletion of lineage-committed hematopoietic cells from the bone marrow [36]. Exposure of thymocytes to gliotoxin at concentrations ranging from 50 nM to 10 μM results in apoptotic cell death, whereas necrosis is induced by concentrations greater than 500 μM [38]. Another *in vitro* study [41] showed gliotoxin to induce apoptosis in concanavalin A-stimulated splenic T-cells and in peritoneal macrophages, with similar results being conferred by exposure to the sporidesmin mycotoxin. At nanomolar concentrations, induction of apoptosis by gliotoxin is apparently due to inhibition of NF-κB activity by prevention of IκB degradation [36].

Gliotoxin causes Ca^{2+} release from mitochondria by reacting with thiol compounds, and the Ca^{2+} release can be prevented by using cyclosporin A [42]. Nevertheless, apoptosis induced by treatment with gliotoxin or dexamethasone, both of which maintain IκB-α expression, was shown to be Ca^{2+} independent [38]. The lethality of gliotoxin is equivalent irrespective of the glutathione status of the target cell [42]. However, death from exposure to the gliotoxin or sporidesmin mycotoxins can be prevented by the presence of Zn^{2+} salts in the culture medium at nontoxic concentrations [41].

It may be useful to incorporate gliotoxin into regimens aimed toward elimination of leukemia and lymphoma cells. Of note, one study [43] showed gliotoxin to also have activity as a farnesyltransferase inhibitor that prevents farnesylation of the $p21^{ras}$ protein, which allows attachment of Ras to the interior cell membrane and is essential for its biological activity. Moreover, oncogenic mutant Ras protein appeared more sensitive than wild-type Ras to inhibition by gliotoxin. Thus, there may be two directions by which gliotoxin can target malignant cells, that is, by inhibition of signalling pathways involving NF-κB and by Ras activation.

Kinase Inhibitors

The mitogen-activated 90-kDa ribosomal S6 kinase ($p90^{rsk1}$) is a downstream substrate of the MAPKK/MAPK signalling pathway [9, 10]. The $p90^{rsk1}$ kinase not only uses ribosomal protein S6 as a target for phosphorylation, but it also has activity as an IκB-α kinase that is required for phosphorylation and subsequent degradation of IκB-α in order to activate NF-κB [10]. Response to mitogenic stimuli can be inhibited using a dominant-negative mutant of $p90^{rsk1}$. Growth hormone (GH) causes phosphorylation of ribosomal protein S6 through activation of $p90^{rsk1}$ and $p70^{s6k}$ kinases, and the activity of the S6 kinases is controlled by their phosphorylation status [44]. Pretreatment of cells with a selective inhibitor of PKC prevents S6 kinase activation by GH, but not by epidermal growth factor. The phosphorylation of the ribosomal S6 protein in response to insulin stimulation is performed by $p70^{s6k}$, whose kinase activity is regulated by the tyrosine-phosphorylated IRS-1, while the IL-1 mimic tetradecanoyl phorbol acetate (TPA)-induced S6 phosphorylation is carried out by $p90^{rsk1}$, whose activation is dependent on the MAPKK/MAPK cascade [9]. Treatment of cells with AG-18, a PTK inhibitor, prevented activation of both the $p70^{s6k}$ and $p90^{sk1}$ kinases and, by this means, may prevent activation of NF-κB in response to TNF-α, thereby leaving the death response free from the counterbalancing NF-κB survival response.

Substances such as CCK, carbachol, and bombesin can activate $p90^{rsk1}$ via increased phosphorylation at very low concentrations (e.g. for CCK the threshold is 10 pM and maximal stimulation occurs with 1 nM) [45]. Activation of CCK appears to be mediated by PKC since TPA activates $p90^{rsk1}$, and the PKC-specific inhibitor GF-109293X strongly inhibits activation of $p90^{rsk1}$ by CCK [45]. Along with the PKC inhibitor, other specific inhibitors had parallel effects on inhibition of $p90^{rsk1}$ and MAPK activities: namely, the PI 3-kinase-specific wortmannin, the MAPK-specific PD98059, and rapamycin [45]. The S6 kinase agonists A23187, bombesin, and PMA activate PI 3-kinase in a wortmannin-sensitive fashion [46]. Inactivation of PI 3-kinase by wortmannin or demethoxyviridin is irreversible [46].

MNK1 is a MAPK that is very homologous to $p90^{rsk1}$ [11]. MNK1 becomes activated when the cells are exposed to TNF-α, IL-1β, IR, or TPA [11], mirroring the response pattern for NF-κB and possibly being involved in its activation. MNK1 is activated by the ERK1 and p38 MAPKs, but not JNK/SAPK. MNK1 activation can be prevented using the MEK inhibitor PD98059 and the p38 MAPK inhibitor SB202190 [11]. MNK1 demonstrates similarities to $p90^{rsk1}$, which is a kinase that phosphorylates the IκB-α inhibitory subunit of NF-κB. The IKK-α and IKK-β

kinases have also been shown to phosphorylate the IκB subunits, thereby targeting them for ubiquitination and degradation and resulting in activation of NF-κB. It would be worthwhile to investigate whether the compounds shown to inhibit $p90^{rsk1}$ and MNK1 activity can also prevent activation of the IKK signalsome, or even if MNK1 and $p90^{rsk1}$ are, in fact, the same as IKK-α and IKK-β. The calphostin C compound may operate by this means, since it has been shown to block activation of NF-κB by TNF-α through preventing IκB-α phosphorylation [29].

A20 Protein

The β isoform of 14-3-3 is a latent co-activator of the Raf-1 kinase in quiescent cells [47]. Activation of Raf-1 by β 14-3-3 resulted in expression of NF-κB-dependent reporter genes *in vivo* [47]. The 14-3-3 proteins contribute to Ras-independent regulatory effects on Raf-1 activity and resultant NF-κB-dependent gene transcription. The A20 protein belongs to a class of Cys2/Cys2 zinc finger proteins that can bind to the η and ζ isoforms of the 14-3-3 protein family [48]. A20 protein that associates with 14-3-3 isoforms had been characterized previously as an inhibitor of NF-κB activation; however, a recent report [48] demonstrated that A20-type proteins may be more broadly utilized. That is, A20 mutants that no longer associate with 14-3-3 proteins could completely inhibit NF-κB activation induced by TNF-α, IL-1β, or PMA.

Bcl-3

Bcl-3 is a proto-oncogene whose product is overexpressed in cells from chronic lymphocytic leukemia (CLL) patients having the t(14;19) gene translocation, and the Bcl-3 protein is a member of the IκB family [49, 50]. The Bcl-3 protein inhibits DNA binding and gene activation by NF-κB/Rel-family heterodimers and p49 and p50 homodimers by sequestering them in the nucleus, as does the IκB complex in the cytosol. Bcl-3 does not inhibit the function of the p65/RelA homodimer and neither does the IκB-α subunit, this being explained by the observation that removal of N-terminal amino acids from the Bcl-3 protein generates a protein almost identical to IκB-α [50]. Transient transfection with the *bcl-3* gene may sensitize target cells to eradication by diverting the localization of Rel factors that have entered the nucleus.

Protease Inhibitors

Activation of NF-κB by various cellular stimuli requires the phosphorylation and subsequent proteolytic degradation of the IκB subunits in their inhibitory complex [17, 51]. A primary pathway for IκB degradation is through post-phosphorylation ubiquitination targeting the subunits for proteolysis by the 26S (1500 kDa) proteasome complex [17]. The proteasome complex that degrades ubiquitinated proteins is formed by association of three subunits: CF-1, CF-2, and CF-3 [52]. The CF-3 factor comprises the 650-kDa multicatalytic protease component. The 250-kDa CF-2 factor is an endogenous inhibitor of the proteasomal activity [21, 52]. The 250-kDa fraction contains a smaller subunit that appears to regulate the inhibitory activity. This factor migrates at 55 kDa in the presence of ATP but shifts to 40 kDa in the absence of ATP, consistent with the removal of ubiquitin. It appears that ATP-dependent ubiquitination of the 40-kDa CF-2 inhibitory subunit regulates assembly and/or proteolytic activity of the 1500-kDa proteasome complex.

Using a wide variety of exogenous protease inhibitors, some of which have specificity for the multicatalytic proteasome complex, it has been demonstrated that degradation of IκB is required for NF-κB activation and that this can be prevented by stabilizing the presence of IκB subunits. For example, LPS stimulates NF-κB activity by inducing phosphorylation and ubiquitin conjugation of IκB, resulting in proteasomal degradation of IκB subunits and nuclear translocation of NF-κB [53]. The calpain inhibitor I decreased LPS-induced translocation of NF-κB via inhibiting by 50% the rapid degradation of IκB-α and by ~100% the delayed degradation of IκB-β [53]. The IκB-ϵ inhibitory subunit has slower degradation kinetics than -α or -β [54], and its presence was not assessed in the just-mentioned report. This section describes the many inhibitors of proteolytic degradation that may be used to block activation of NF-κB, with the intent of enabling sensitization of malignant cells to therapeutic eradication.

Inactivation of the proteasome using a proteasome-specific protease inhibitor is one means by which IκB degradation could be prevented. The active component of the endogenous ATP-stabilized CF-2 inhibitor of the proteasome complex has been defined as ALAD [55]. ALAD is also the second enzyme in the heme synthesis pathway, and both CF-2 and ALAD have demonstrated similar dehydratase and proteasome-specific inhibitory properties. "Gene sharing" would explain the dual role of this protein in the ubiquitin-dependent proteolytic pathway and in heme synthesis and could account for the inexplicably abundant expression of ALAD in cells noted in earlier studies.

Lactacystin is a cell-permeant protease inhibitor that is specific for the proteasome [56–59]. Lactacystin inhibits the proteasome by acylation [59]. Lactacystin is inactive until converted to clasto-lactacystin β-lactone by lactonization. The β-lactone binds to the sulfhydryl of glutathione, and this thioester adduct is functionally inactive. The lactathione can be converted back to active β-lactone that can inhibit the proteasome. Lactathione formation concentrates inhibitor inside the cell and serves as a reservoir for prolonged release and inhibition of the proteasome [59].

Degradation and turnover of the tumor-suppressor protein p53 result from ubiquitination and can be prevented by a proteasome inhibitor [60]. Conjugates of p53 and ubiquitin were detectable in untreated and IR-exposed cells. The inhibition of proteasome function stabilized and in-

creased the presence of p53 in normal cells and those expressing the human papillomavirus 16 E6 oncoprotein. The cytostatic lactacystin proteasome inhibitor induces $p21^{cip1}$ cyclin-dependent kinase inhibitor expression in cancer cells regardless of their p53 status [60, 61]. Wild-type p53 cells increase p21 mRNA transcription, and mutant p53 cells have increased p21 protein half-life when exposed to lactacystin.

Treatment of cells with micromolar concentrations of the cell-permeant aldehydic proteasome inhibitor Cbz-Ile-Glu-(O-t-Bu)-Ala-leucinal (PSI) inhibits chymotrypsin-like activity of the proteasome [17, 62]. PSI prevents activation of NF-κB in response to TNF-α or the phosphatase inhibitor okadaic acid (OA) through inhibition of IκB degradation [17]. Newly phosphorylated IκB accumulated in TNF- and OA-stimulated cells but remained associated with NF-κB. Whereas PSI prevented protein degradation in stimulated or unstimulated cells, the nonproteasomal calpain protease inhibitor Cbz-Leu-leucinal did not, at the concentrations tested [17, 62].

MG132 is a proteasome inhibitor that extends the half-life of multi-ubiquitinated proteins *in vivo* [63]. LPS and silica both induce degradation of IκB-α, and MG132 prevents LPS-induced, but not silica-induced, IκB degradation and NF-κB activation [63]. Interferon-induced STAT activity is stabilized *in vivo* by a proteasome inhibitor [64], and MG132 stabilizes IL-2-induced DNA binding and tyrosine phosphorylation of STAT5 even though it does not become ubiquitinated [65]. PTPase activity negatively regulates STAT activity, and the sustained activation of STAT5 can be blocked by a PTK inhibitor, implying that Jak kinases are degraded by proteasomes and that this can be blocked by MG132 [65].

Other compounds that appear to be proteasome-specific protease inhibitors include aprotinin [66], ZLLnV-CHO, ZLLL-CHO [57], and *N*-benzyloxycarbonyl-Ile-Glu-(*O-t*-Bu)-Ala-leucinal [67]. Alternative uses for proteasome inhibitors may be found in therapy for viral infections. The *tax* gene product of HTLV-1 induces nuclear localization and gene function of NF-κB by phosphorylation followed by degradation of IκB-α [68]. The Tax-mediated activation of NF-κB can be prevented using a proteasome inhibitor that causes accumulation of a phosphorylated form of IκB-α [68].

There are several ubiquitin C-terminal hydrolases (UCHs) that cleave and recycle ubiquitin from protein conjugates being processed by the proteasome [69]. Sulfhydryl-blocking agents like iodoacetamide and ubiquitin-aldehyde (Ubal) block UCH activity. Ubal concentrations in the range of 3–10 μM inhibit the isopeptidases that normally disassemble "unanchored" polyubiquitin chains that remain after substrate degradation by the proteasome [69, 70]. The prevention of ubiquitin cleavage using Ubal inhibits protein conjugate degradation.

Some protease inhibitors that are not specific for the proteasome have been found to prevent IκB degradation and activation of NF-κB. This could be explained by the recent description of another pathway of IκB degradation that is different from the one involving proteasomes [71]. When lysosomal protein degradation is inhibited with protease inhibitors, IκB levels are increased markedly. The degradation of IκB inside lysosomes results from direct transport of the protein through the lysosomal membrane, similar to the pathway described for other cytosolic proteins. Thus, there appear to be at least two pathways for degradation of IκB proteins, each of which could be affected by the appropriate proteasome- and nonproteasome-specific protease inhibitors.

Calpains regulate gene expression through processing of NF-κB [23]. DNA-binding activity can be abolished by proteolysis of NF-κB with μ- and m-calpains. Proteolysis of NF-κB by calpains is prevented using calpastatin, calpain inhibitor I, or a proteasome inhibitor [23]. The finding that calpain inhibitor I and a proteasome inhibitor protect NF-κB from degradation is consistent with their roles in preventing degradation of IκB subunits, thereby preventing activation of NF-κB ([29, 53] and see below).

The nonproteasomal calpain protease inhibitor *N*-acetyl-Leu-Leu-norleucinal stabilizes IκB-α and prevents activation of NF-κB [2, 28]. Other calpain-selective inhibitors include the cell-permeant ZLLY-DMK, which can prevent transit of cells into S-phase, and *N*-benzyloxycarbonyl-Leu-leucinal [57]. A caveat is that the lysosome inhibitor chloroquine actually enhances NF-κB activity by inhibiting resynthesis of IκB-α [72].

CONCLUSIONS

NF-κB activity affects cell survival and determines the sensitivity of cancer cells to cytotoxic agents as well as IR. Preventing the protective function of NF-κB may result in chemo- and radio-sensitization of cancer cells. Therefore, NF-κB has emerged as one of the most promising molecular targets in rational drug design efforts of translational cancer research programs.

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