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Review

Cell penetrating peptide inhibitors of Nuclear Factor-kappa B

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Abstract. The nuclear factor kappa B (NF-κB) transcription factors are activated by a range of stimuli including pro-inflammatory cytokines. Active NF-κB regulates the expression of genes involved in inflammation and cell survival and aberrant NF-κB activity plays pathological roles in certain types of cancer and diseases characterized by chronic inflammation. NF-κB signaling is an attractive target for the development of novel anti-inflammatory or anti-cancer drugs and we discuss here how the method of peptide transduction has been used to specifically target NF-κB. Peptide transduction relies on the ability of certain small cell-penetrating peptides (CPPs) to enter cells, and a panel of CPP-linked inhibitors (CPP-Is) has been developed to directly inhibit NF-κB signaling. Remarkably, several of these NF-κB-targeting CPP-Is are effective *in vivo* and therefore offer exciting potential in the clinical setting.

Keywords. Nuclear Factor kappa B, I kappa B Kinase, cell penetrating peptide, peptide transduction, signal transduction.

Introduction

Diverse stimuli activate the nuclear factor kappa B (NF-κB) transcription factors. These include pro-inflammatory cytokines, signals that activate innate and adaptive immune receptors, and various cellular stresses [1, 2]. In turn, NF-κB regulates the expression of many genes involved in responses ranging from inflammation and innate and adaptive immunity, to cell growth, survival, and proliferation [1 – 3]. Under normal circumstances, NF-κB activation occurs rapidly and transiently; dysregulated or constitutive NF-κB activity, however, has been functionally linked to the development of diseases, including those characterized by chronic inflammation, as well as autoimmunity and certain cancers [2 – 5]. In light of this important detrimental role of aberrant NF-κB signaling, a major goal of many pharmaceutical enterprises is the development of therapeutically effective, highly specific inhibitors of NF-κB. Over seven hundred natural and synthetic compounds that block NF-κB activity have been identified and a number of drugs targeting this pathway have been described [5, 6]. Because most of these compounds either lack specificity or disrupt the normal physiological functions of NF-κB, they are less than ideal candidates for clinical use.

As our understanding of the complexity of NF-κB signaling expands, promising novel molecular targets

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for the development of specific inhibition strategies continue to emerge. For example, the demonstration that NF-κB activation occurs through two major transduction mechanisms, named the classical and non-canonical pathways, that utilize distinct upstream signaling components has revealed novel targets that could be exploited to develop pathway-specific drugs [2, 3, 7 – 9]. In addition to the obvious therapeutic value of such selective reagents, the ability to directly inhibit specific signaling components in the many pathways leading to NF-κB activation will greatly assist basic researchers trying to dissect these mechanisms *in vitro* and *in vivo* in models of disease.

A method for introducing exogenous bioactive macromolecules into cells has been used effectively to specifically inhibit NF-κB signaling. Named peptide transduction, this methodology not only provides researchers with a convenient means to manipulate NF-κB signaling in cells and *in vivo*, but also may aid in identifying and validating novel selective targets for the development of a new generation of highly specific drugs targeting NF-κB. Peptide transduction hinges on the ability of an expanding group of small peptides to cross the plasma membrane and enter cells [10 – 14]. These are named cell-penetrating peptides (CPPs) and include both naturally occurring and synthetic sequences. The utility of this approach for the detailed study of signal transduction pathways rests in the fact that CPPs can be conjugated to a wide range of bioactive cargos, allowing large exogenous macromolecules that would otherwise be excluded from cells to rapidly enter through a process called membrane transduction. These cargos are then able to immediately exert their biological effects directly on their molecular targets within the cell. This "Trojan Horse" approach has been used extensively to manipulate signal transduction mechanisms *in vitro* and *in vivo* and a number of CPP-linked inhibitors (herein referred to as CPP-Is) that directly target NF-κB signaling have been developed.

In the following section we will briefly introduce the basic principals of peptide transduction, focusing our discussion on the CPPs that have been used to study NF-κB signaling. We will then discuss NF-κB signaling and describe in depth the CPP-Is that have been developed to target NF-κB.

Peptide transduction

The ability to manipulate proteins in living cells is a crucial method for studying protein function and for validating potential drug targets. Some approaches require the introduction of bioactive material into cells. This material may include DNA constructs encoding mutated versions of effector proteins or reagents, such as antisense or short-interfering RNA (siRNA), to knock down gene expression. Various techniques overcome the natural resistance of the plasma membrane to exogenous material and the most widely used of these are lipid-based transfection, viral vectors, electroporation, and microinjection. Despite the almost universal application of these techniques, they have limitations. For example they can be (i) inefficient and result in low levels of transfection; (ii) cytotoxic or harsh and cause excessive cell loss; (iii) complex in that they might require specialized equipment or reagents and involve extensive optimization of conditions; (iv) are often not effective for primary or non-dividing cells; (v) capable of drastically altering cell activation state in their own right; and (vi) unreliable or not applicable due to organismal toxicity for *in vivo* studies of protein function or target validation.

Peptide transduction offers an attractive alternative approach for the introduction of bioactive reagents directly into living cells where they can immediately exert their effects. Biophysical, biochemical, and *in vitro* and *in vivo* studies demonstrate that peptide transduction largely overcomes the problems associated with the more traditional transfection methods. Thus, CPP-mediated transduction is generally non-toxic within the effective concentration ranges, it can rapidly deliver a diverse assortment of molecular cargos into all cell types tested (including primary and nondividing cells), and, most importantly, it is highly effective *in vivo* where it can direct bioactive cargo into all tissues including the brain [10 – 15].

Cell-penetrating peptides (CPPs)

Naturally occurring and synthetic CPPs, fall into three classes based upon their biophysical properties: cationic (so named for the presence of arginine or lysine residues), hydrophobic, and amphipathic peptides (Table 1). The distinct characteristics of these CPPs facilitate their uptake across the plasma membrane and the best studied in this regard are the cationic peptides that contain a number of positively charged arginine or lysine residues. Even synthetic CPPs of seven to 11 residues composed solely of arginine (Poly-Arg) or lysine (Poly-Lys) enter cells, underscoring the importance of cationic residues for uptake [10, 16, 17]. Of the three classes of CPPs, members of both the cationic and hydrophobic groups have been extensively used to manipulate NF-κB signaling. To date, however, none of the amphipathic CPPs has been used to deliver cargo targeting NF-κB

Table 1. Commonly used CPPs. The CPPs most commonly used for peptide transduction belong to three separate groups: Cationic, Hydrophobic and Amphipathic. The sequences of the best-characterized members of these groups are shown. Asterisks denote the CPPs that have been used in studies of NF-κB signaling (see Table 2). The residues of PTD-5 derived from the HIV-1 TAT sequence are underlined. Abbreviations: PTD, peptide transduction domain; MTS, Membrane translocating sequence from the h-region of the Kaposi' FGF signal sequence.

CPP	Sequence	[Ref.]
Cationic		
*HIV-1 TAT fragment (47 – 57)	YGRKKRRQRR	[20]
**Drosophila* Antennapedia (43 – 58)	RQIKIWFQNRRMKWKK	[22]
*Poly-arginine (synthetic)	R*n* (*n* = 7 – 11)	[16]
*Poly-lysine (synthetic)	K*n* (*n* = 8 – 10)	[17]
*PTD-5 (synthetic)	RRQRRTSKLMKR	[16]
Hydrophobic		
*MTS	AAVALLPAVLLALLAP	[26]
Amphipathic		
Transportan (synthetic)	GWTLNSAGYLLGKINLKALAALAKKIL	[14]
KALA (synthetic)	WEAKLAKALAKALAKHLAKALAKALKACEA	[14]

signaling and these will not be further discussed here [see [11 – 15, 18] for reviews].

Five CPPs and their derivatives have been used in studies of NF-κB signaling (Table 1). These include the first membrane-permeable peptide identified, which was the sequence located between residues 47 and 57 of the human immunodeficiency virus (HIV)-1 TAT protein that was necessary and sufficient for cell permeation of the entire TAT protein [19, 20] and was the minimal domain required for traversing cell membranes [21]. This TAT fragment is now widely utilized as a CPP, including for the transduction of distinct peptide or protein cargos that block components of the NF-κB pathway (Table 2). A second, widely used cationic CPP utilized to study NF-κB signaling is derived from the third α-helix of the *Drosophila* homeodomain transcription factor Antennapedia. The segment between residues 43 and 58 of Antennapedia is sufficient for membrane transduction of the entire protein [22, 23] and is commonly named AntP (or penetratin). A third cationic CPP is the synthetic peptide transduction domain (PTD)-5 [16], which enters some cell types more efficiently than TAT or AntP. The transducing ability of PTD-5 requires the positively charged RRQRR motif, which is derived from the parent TAT fragment (underlined in Table 1). The last of the cationic CPPs used to deliver cargo targeting NF-κB signaling are synthetic poly-arginine or poly-lysine homopolymers that have been reported to be more efficient than either TAT or AntP for *in vivo* delivery [16, 24, 25].

The only hydrophobic CPP used to deliver cargo to target the NF-κB pathway is derived from the hydrophobic region (h-region) of the signal sequence of Kaposi fibroblast growth factor (kFGF, also known as FGF-4) and is named the membrane translocating sequence (MTS) [26]. Although MTS is considered less effective than the cationic peptides [14], it remains a frequently used CPP for signal transduction research.

The mechanisms of transduction are distinct for each class of CPP. Initially, uptake of cationic CPPs was thought to be energy-, receptor-, and endocytosis-independent [10, 19, 20, 22, 23, 27, 28], however it is now clear that endocytosis or macropinocytosis is involved [29 – 33]. The initial association of TAT, AntP, poly-R, or poly-K with the plasma membrane occurs through an ionic interaction between the positively charged residues in the peptides and negatively charged membrane phospholipids and proteoglycans, including heparan sulfate [16, 30, 34, 35]. In contrast, the hydrophobic CPP MTS does not rely on endocytosis for in membrane transduction [26, 32]. Studies of MTS and other related hydrophobic signal sequence CPPs have suggested that the secondary conformation is important for transduction and a current model is that MTS forms an alpha helical hairpin that interacts with membrane lipids, then unloops to a transmembrane conformation, penetrates the phospholipid bilayer, and carries the cargo into the cell [36].

CPPs transduce most types of cells in the absence of cytotoxicity, although high concentrations (>100μM) of TAT and AntP have been shown to be toxic to some cell types [37, 38]. When conjugated with bioactive cargo, cytotoxicy that is dependent upon either the CPP used, the length and nature of the cargo molecule, or the method used to couple the cargo to

Table 2. CPP-inhibitors of the classical NF-κB pathway. Five targets within the classical NF-κB pathway have been successfully disrupted using CPP-Is (left; see also Fig. 3). The NLS inhibitors block the nuclear localization of several transcription factors including NF-κB however the remaining CPP-Is exhibit strong specificity for their targets within the NF-κB pathway (see text and references for details). Sequences of the separate CPPs are shown in Table 1. The residues within some of the cargo sequences that have been mutated to generate inactive mutant versions of the CPP-Is are underlined. Both of the CPP-Is targeting IκBα are fusions of the CPP with mutant versions of the IκBα protein. TAT-srIκBα (sr, super repressor) contains alanines at the critical phosphorylation sites (Serr32 and Ser36) within the N-terminus whereas MTS-IκBα(ΔN) lacks the first 36 N-terminal amino acids. Both fusions act as dominant negatives by preventing phosphorylation of wild-type IκBα by the IKK complex. The references cited are the original reports for each CPP-I.

Target	CPP-Inhibitor (CPP-I)	Cargo Sequence	[Ref.]
Nuclear Localization Sequence (NLS)	MTS-p50 NLS (SN50)	VQRKRQKLMP	[26]
	MTS-thiazidolino-p50 NLS (ScN50A)	CYVQRKRQKLMP	[41]
	MTS-thiazidolino-p50 NLS (ScN50B)	CYVQRKRQKLMP	[41]
	MTS-SV40 NLS (BMS-205820)	PKKKRKV	[104]
	MTS-c-myc NLS (BMS-214572)	AKRVKL	[105]
	AntP-p50 NLS (PN50)	VQRKRQKLMPC	[102]
NEMO Binding Domain (NBD)	AntP-NBD	TALDWSWLQTE	[64]
	TAT-NBD	TALDWSWLQTE	[110]
	PTD-5-NBD	TALDWSWLQTE	[111]
	8K-NBD	TALDWSWLQTE	[167]
NEMO Oligomerization	AntP-NEMO-CC2	SKGMQLEDLRQQLQQAEEALVAKQELIDKLKEEAEQHKIV	[68]
	AntP-NEMO-LZ	LKAQADIYKADFQAERHAREKLVEKKEYLQEQLEQLQREFNKL	[68]
	R7-NEMO-LZ	LKAQADIYKADFQAERHAREKLVEKKEYLQEQLEQLQREFNKL	[24]
p65 Phosphorylation	AntP-p65-P1	QLRRPSDRELSE	[182]
	AntP-p65-P6	NGLLSGDEDFSS	[182]
IκBα	TAT-srIκBα	Full length IκBα with Ser32 and Ser36 mutated to alanine.	[184]
	MTS-IκBα (ΔN)	IκBα mutant lacking residues 1-36	[186]

the CPP (see below) has been reported for high concentrations of each of the separate classes of CPPs [37 – 39]. Furthermore, certain CPP-cargo combinations may be cytotoxic for some cell types but well tolerated by others [37 – 39]. In this regard, we have consistently observed that treatment of the Jurkat T cell line with AntP leads to cargo-independent cell death even at low concentrations of the peptide; we have not, however, observed similar toxicity in any other cell line or primary cell type that we have tested (unpublished observations). Although the toxic effects on immune cells in culture may only occur in certain cell lines, caution must clearly be taken in distinguishing effects of CPP-Is that are target-specific and effects that may be due to nonspecific toxicity. Consequently, any strategy involving the use of CPPs to deliver bioactive cargo should first test for toxicity using a range of CPPs, distinct coupling methods and a full range of concentrations of the peptides before conclusions regarding target-specific effects can be drawn.

The range of bioactive cargo delivered by CPPs

CPPs can deliver a range of diverse cargos and influence many intracellular processes [12, 13, 18, 40]. The CPPs in Table 1 have been successfully used to deliver cargos ranging in size from small therapeutic molecules, such as doxorubicin, to liposomes over 200 nm in diameter [13]. Other cargos that have been delivered by CPPs include antisense oligo-nucleotides, siRNAs, plasmids, viral particles and 40nm metallic beads [12, 13, 18, 40]. Three methods are commonly used to conjugate a bioactive cargo with a CPP (Fig. 1): (i) chemical synthesis of chimeric fusions of cargo peptides with the CPPs, (ii) covalent attachment of the CPP and cargo by a chemical linker and (iii) cloning and bacterial expression of recombinant proteins using plasmids harboring the CPP as a "tag" [40]. Due to the relative ease of synthesis and the nature of the separate targets, most NF-κB studies have utilized the first of these approaches to generate tandem fusions of the CPP with the appropriate peptide cargo. However, covalent linkage using a thiazolidine ring as the bridging moiety has also been used for delivering peptides targeting the NF-κB pathway [41]. The third method of generating CPP-tagged recombinant proteins through bacterial expression and protein purification is most commonly used to generate CPPs conjugated to whole proteins. CPPs have been used extensively to deliver small peptides and whole proteins targeting diverse signal transduction pathways and it is this peptide- and

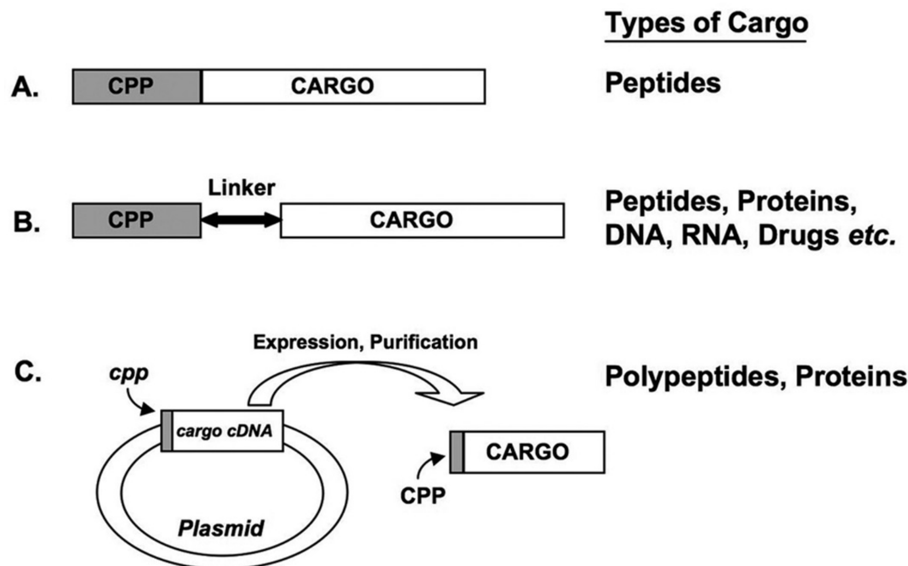

Figure 1. Methods of generating CPP-Is. Three methods are commonly used to conjugate a bioactive cargo with a CPP and each has been employed to generate CPP-Is targeting the NF-κB pathway. The types of cargos typically delivered using each of these methods are described (right). (*A*) Chemical synthesis of chimeric fusions of the cargo with the CPPs. This technique has been the most extensively method employed to generate CPP-Is targeting the NF-κB pathway [26, 64, 68, 182]. (*B*) Covalent attachment of the CPP and cargo via a chemical linker such as a cysteine disulfide bridge or a thiazolidine ring. This latter linker has been used to generate a CPP-I targeting NF-κB nuclear translocation [94]. (*C*) Cloning and bacterial expression of recombinant proteins using plasmids harboring the CPP as a "tag" has been used to deliver mutated forms of IκBα to block the NF-κB pathway [183 – 186].

protein-transducing capability of CPPs that has been exploited to target the NF-κB pathway.

CPPs function *in vivo*

A remarkable feature of peptide transduction technology is the ability of CPPs to effectively deliver bioactive cargo *in vivo*. Work from several laboratories demonstrated that TAT-mediated delivery of β-galactosidase (β-gal) *in vivo* was effective with β-gal activity detected in all tissues including the brain after intravenous (i. v.) [42], intraperitoneal (i. p) [43], or oral administration [44] in mice. Furthermore, comparison of TAT- and viral-mediated transduction of β-gal to the rat salivary gland revealed that 100% of cells were transduced by TAT, whereas only 30 to 50% expressed β-gal following viral delivery [45 – 47]. TAT has also been used to deliver physiologically relevant cargos targeting signaling mechanisms *in vivo*, thereby laying the groundwork for clinical investigation of CPP-Is in animal models of disease [48 – 50]. Although the majority of published *in vivo* studies have used TAT as the CPP, other CPPs are also effective. Cre recombinase has been delivered *in vivo* by fusion to MTS and this resulted in excision in transgenic mice of loxP-flanked gene segments in all tissues examined [51]. Furthermore, AntP has been utilized *in vivo* to transduce peptide cargos that functioned to ameliorate symptoms in several disease models in mice [52]. Taken together, these reports establish CPP-mediated protein and peptide transduction as a powerful approach to block signaling pathways in animal models of disease.

CPP inhibitors of NF-κB signaling

NF-κB signaling. NF-κB proteins are a family of five structurally related transcription factors named p65 (RelA), RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2) [1 – 3]. These proteins homo- or heterodimerize to generate either transcriptionally active (for example, p50:p65, p52:RelB) or repressive (for example, p50:p50) versions of NF-κB. Each of the NF-κB subunits contains a highly conserved Rel homology domain (RHD) that facilitates DNA binding, dimerization, and interaction with the inhibitory IκB proteins. RelB, p65, and c-Rel are each translated in their mature forms; whereas p50 and p52 are the processed NH_2-termini of the longer precursor proteins p105 and p100, respectively. Constitutive processing of p105 maintains a constant cellular pool of p50; whereas p100 processing to p52 occurs only in response to a specific subset of signals.

In resting cells, NF-κB proteins are maintained inactive in the cytosol through interaction with the IκB proteins that mask a nuclear localization se-

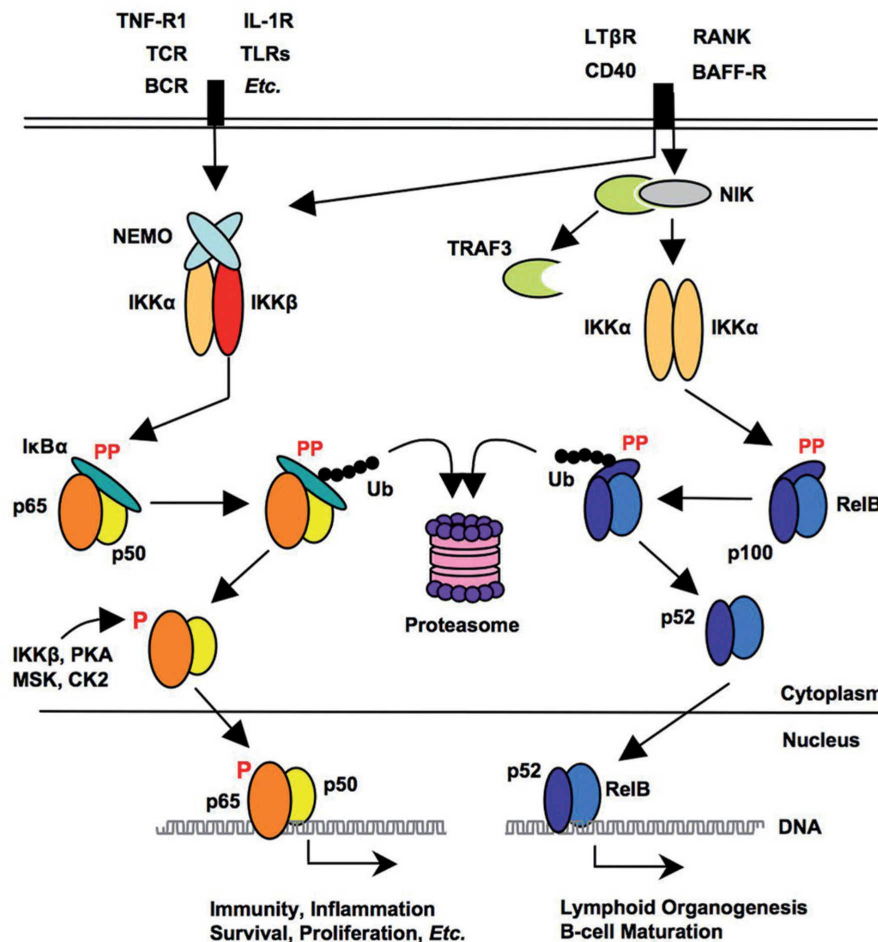

Figure 2. The classical and non-canonical NF-κB pathways. The classical pathway (left) is activated by many stimuli including the pro-inflammatory cytokines IL-1 and TNF, antigen receptor (TCR, BCR) ligation and signaling from the TLRs. This pathway requires NEMO, and for most stimuli IKKβ is the critical kinase that phosphorylates the IκB proteins, leading to their ubiquitin-dependent proteasomal degradation. The free classical NF-κB complexes (typified by p50:p65) then migrate to the nucleus to regulate the expression of genes involved in responses including immunity, inflammation and cell survival. The non-canonical pathway (right) is activated by a subset of signals including ligation of LTβR, CD40 and BAFF-R, and requires NIK-dependent IKKα activation. It has not yet been formally established whether the IKKα that functions in the non-canonical pathway exists in a separate complex containing only IKKα (as depicted here and in most models), or if it is the IKKα within the IKKα-IKKβ-NEMO holocomplex that is the effector kinase of non-canonical signaling. Activated IKKα phosphorylates the COOH-terminus of p100 complexed with RelB leading to its processing to p52 to generate p52:RelB complexes. This pathway regulates the expression of chemokine and cytokine genes involved in lymphoid organogenesis and B cell maturation. The molecular interactions and the many signaling events that occur up- and downstream of classical and non-canonical NF-κB activation are discussed in detail in several recent reviews [2, 7 – 9]. In addition, the website maintained by Dr. Thomas Gilmore's group (www.nf-kb.org) is an excellent resource for all aspects of NF-κB biology.

quence (NLS) in the NF-κB subunits. IκBs themselves belong to a family of proteins hallmarked by multiple ankyrin repeat domains that facilitate their binding to the RHDs of the NF-κB proteins. The prototypic IκB protein is IκBα and the family also contains IκBβ, IκBε, and the COOH-termini of p100 and p105 that function as IκBs in certain NF-κB complexes [2, 3]. NF-κB activation is initiated by phosphorylation of the IκBs leading to their ubiquitination and then degradation by the proteasome. For p100 and p105, only their IκB-like COOH-termini are degraded, leaving the NH_2-termini (p52 and p50 respectively) intact. Once freed from IκB, NF-κB translocates to the nucleus where it binds to target gene promoters and regulates gene expression. The kinases that phosphorylate IκB proteins are components of the IκB-kinase (IKK) complex that consists of two catalytic subunits named IKKα (also known as IKK1) and IKKβ (also known as IKK2) and a noncatalytic regulatory component named NEMO (NF-κB essential modulator, which is also known as IKKγ) [7 – 9] Intriguingly, genetic studies targeting each of the IKK complex subunits have delineated two mechanistically distinct pathways leading to the activation of separate NF-κB proteins that regulate discrete panels of target genes and physiological responses [3, 7 – 9].

By far the best studied of these mechanisms is "classical" NF-κB signaling and it is this pathway that is rapidly and transiently activated by most known NF-κB inducers, including the pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukin 1 (IL-1), as well as engagement of innate [that is the Toll-like receptors (TLRs)] and adaptive [the T cell receptor (TCR) and the B cell receptor (BCR)] immune receptors (Fig. 2) [2, 9]. Classical NF-κB activation requires signal-induced, site-specific phosphorylation of IκBα on two serine residues (S32 and S36 in human IκBα) in its NH_2-terminus. This phosphorylation triggers the subsequent ubiquitination and proteasomal degradation of IκBα, releasing NF-κB proteins from their cytoplasmic retention. The most abundant NF-κB species activated in this manner are p50:p65 heterodimers that are complexed with IκBα in most cell types and are rapidly activated following stimulation. In addition to IκBα phosphorylation, classical pathway activation also requires phosphorylation of p65 (Fig. 2) and several kinases mediate this, including IKKβ, the protein kinase A (PKA) catalytic subunit, mitogen- and stress-activated protein kinase (MSK), and casein kinase 2 (CK2) [53 – 56]. Phosphorylation of p65 is absolutely critical for its transcriptional activity because phosphorylation allows p65 to interact with transcriptional cofactors, such as the histone acetyltransferases CBP or p300, collectively referred to as CBP/p300 [56]. Classical NF-κB activity regulates the expression of many genes involved in immune, inflammatory, and survival responses, including those encoding cytokines (for example, IL-1, IL-2, IL-6, TNF), chemokines (for example, CXCL8, CCL2, CCL3), leukocyte adhesion molecules (for example, E-selectin, ICAM-1, and VCAM-1), and antiapoptotic proteins (for example, Bcl2, Bcl-X_L, XIAP). Expression of these genes is normally tightly regulated by the transient kinetics of classical NF-κB signaling [2]. However, dysregulated aberrant NF-κB activity in diseases such as chronic inflammation and cancer leads to sustained expression of classical pathway-dependent genes and underlies the pathophysiological role of classical NF-κB in these conditions [4, 5].

Studies with cells lacking each of the IKK complex components have established a model for classical NF-κB signaling in response to most stimuli that is dependent upon IKKβ and NEMO (Fig. 2) [57 – 61]. IKKβ is the catalytic component of the IKK complex that directly phosphorylates IκBα leading to its ubiquitin-dependent degradation [59]. However, certain inducers including RANK-L (receptor activator of NF-κB ligand) and IL-1 can activate the classical pathway in the absence of IKKβ, suggesting that IKKα and NEMO may in fact transduce a subset of classical signals [62, 63]. NEMO is absolutely crucial for all classical NF-κB signaling and cells lacking NEMO fail to respond to any inducers of the classical pathway [60, 61]. Within the IKK holocomplex NEMO associates with both IKK subunits at a small region within their COOH-termini named the NEMO binding domain (NBD), suggesting that NEMO regulates the activity of each of the IKKs [64, 65]. Precisely how NEMO functions remains unclear; however, through a ubiquitin-binding domain (UBD), NEMO interacts with Lys^{63}-ubiquitinated adaptor proteins in several pathways [66, 67]. This interaction recruits the IKKs to receptor adaptor complexes where they may be activated by upstream kinases or by proximity-induced trans-autophosphorylation. NEMO also oligomerizes through a minimal oligomerization domain and this is critical for signal-induced activation of the IKK complex in the classical pathway [24, 68 – 71]. The precise role of oligomerization is not known but it is possible that it facilitates crosstalk between separate IKK subunits brought into close proximity by NEMO-NEMO interactions.

In contrast to classical NF-κB signaling, the second NF-κB pathway does not require either IKKβ or NEMO. This mechanism is named the alternative or noncanonical pathway and is dependent on IKKα (Fig. 2) [72 – 75]. The noncanonical NF-κB pathway only targets p100:RelB heterodimers which are maintained inactive by the IκB-like COOH-terminus of p100 [75]. IKKα is the key effector kinase in the noncanonical pathway where it specifically phosphorylates the p100 COOH-terminus inducing ubiquitin-dependent proteasomal processing to p52. A second kinase upstream of IKKα named NIK (NF-κB-inducing kinase) that phosphorylates and activates IKKα [76 – 78] is also critical for noncanonical signaling, and p100 processing is absent in alymphoplasia mice that carry a mutated NIK gene ($NIK^{aly/aly}$) [77, 78]. In resting cells NIK is rapidly turned over through ubiquitination by TRAF3, which causes NIK degradation [79]. Activation of noncanonical pathway-inducing receptors sequesters TRAF3 leading to increased abundance of NIK and activation of IKKα [79].

The noncanonical pathway is activated by a subset of TNF receptor family members, including the lymphotoxin-β receptor (LTβR), CD40, RANK, and BAFF-R (B cell-activating factor receptor) (Fig. 2, right). Most stimuli that activate the classical pathway (such as TNF, IL-1, TCR, BCR) do not activate noncanonical signaling, whereas activation of LTβR, CD40, RANK, or BAFF-R can activate either the noncanonical or the classical mechanism. The major functions of noncanonical NF-κB signaling are regulation of B cell survival and maturation and peripheral

lymphoid organogenesis [72, 75], and mice lacking each of the components of the pathway (NIK, IKKα, p100 or RelB) exhibit profound defects in these developmental processes [7]. Reflecting these functions, the few genes that are confirmed targets of p52:RelB are BAFF, the ligand for BAFF-R, and the chemokines CXCL12, CXCL13, CCL19, and CCL21, which function during lymphoid organogenesis [7, 72, 74, 75].

In summary, NEMO and IKKβ are required for classical NF-κB activation in response to most inducers of this pathway. In contrast, neither NEMO nor IKKβ function in the noncanonical pathway, which depends on NIK and IKKα (Fig. 2). Because the classical pathway has critical roles in regulating immune and inflammatory responses, as well as cell survival, it is a highly promising target for the development of drugs aimed at blocking the detrimental role of NF-κB in diseases, such as chronic inflammation and cancer. CPP-Is specifically targeting the classical NF-κB pathway have been developed (Fig. 3) and are effective *in vitro* and, in some cases, *in vivo* using animal models of disease.

To date peptide transduction approaches have only been applied to studies of the classical pathway and the genes and pathophysiological responses it regulates. However, given the potential clinical importance of developing NF-κB pathway-specific blocking strategies, we anticipate that noncanonical pathway-targeting CPP-Is will emerge in the near future. The CPP-Is discussed in detail in the following sections were specifically developed to block classical signaling by either disrupting the IKK complex or by inhibiting critical events downstream of IKKβ (Table 2; Fig. 3). In addition, several peptides that target upstream intermediates in the NF-κB pathway or other signaling mechanisms, such as the ERK and JNK pathways, have also been developed. These IKK-proximal CPP-Is are described briefly.

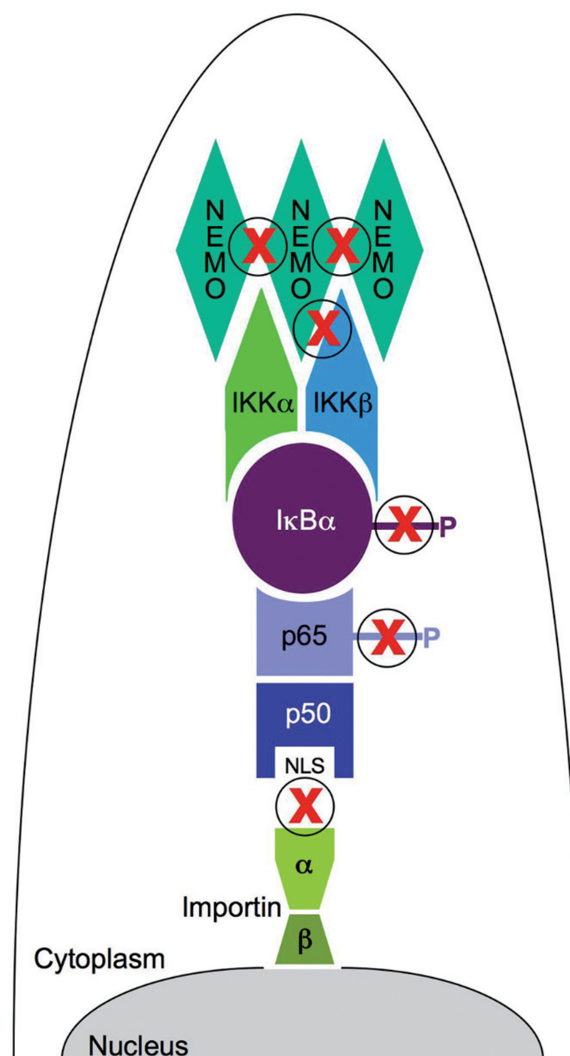

Figure 3. CPP-Is that disrupt the classical NF-κB pathway. The cytoplasm of a cell is shown and individual proteins are depicted in different colors. The nucleus is shaded and a nuclear pore indicated by a break in the line representing the nuclear membrane. CPP-Is targeting the classical NF-κB pathway are denoted by the circled red "x" and these are each discussed in detail in the text. The CPP-I between importin and the p50 NLS represents the nuclear localization inhibitors SN50, PN50 and the double NLS construct. The CPP-I between NEMO and IKKβ represents the NBD peptide that blocks association of NEMO with the IKKs and the two CPP-Is between NEMO subunits depict peptides that disrupt NEMO oligomerization. The CPP-I on p65 represents peptides mimicking the phosphorylation sites on p65 and the CPP-I on IκBα depicts two constructs that inhibit NF-κB activation by blocking IκBα phosphorylation and degradation.

CPP-Is targeting IKK-proximal signaling

Several CPP-linked peptide inhibitors of NF-κB signaling that function upstream of IKK complex activation have been described. One group of these peptides directly targets the TLR / IL-1 family of innate immune receptors or their immediately distal adaptor proteins. In a recent report, Toshchakov and co-workers generated three separate AntP fused peptides spanning the highly conserved BB loops within the TIR (Toll-interleukin 1 receptor) domains in the cytoplasmic tails of TLRs 1, 2, 4 and 6 [80]. BB loops are the docking sites for downstream adaptor proteins and, consistent with this, the TLR2- and TLR4-BB peptides blocked NF-κB activation in macrophages in response to LPS and two lipopeptides (P2C and P3C). Intriguingly however, although TLRs 1 and 6 form heterodimers with TLR2, a peptide spanning the identical BB domains in these receptors (TLR1/6-BB) did not block NF-κB signaling in response to any agonist. These findings therefore revealed distinct but as yet unclear roles for the TLR-

BB domains in regulating the unique signaling functions of these individual receptors [80].

Two separate CPP-Is targeting adaptor proteins that associate with the TLR / IL-1 receptor family have also been described. The first of these is a peptide directed against TIR domain-containing adaptor protein (TIRAP) that is recruited to TLR4 following LPS stimulation [81]. Horng and colleagues demonstrated that a fusion of the TLR4 interaction domain of TIRAP with AntP (AntP-TIRAP) blocked LPS-induced NF-κB activation in a murine monocytic cell line and primary dendritic cells [81]. AntP-TIRAP has also been shown to function *in vivo*, where it blocked LPS-induced innate immune responses in the lungs of mice [82]. Importantly, although AntP-TIRAP effectively blocked LPS signaling, it had no effect on TLR9-induced NF-κB activation [81]. This therefore demonstrates that selective targeting of receptor-specific adaptor proteins using CPP-Is can be used to block NF-κB activation in a pathway-specific manner.

The second CPP-I targeting TLR / IL-1 receptor signaling adaptor proteins was derived from the BB sequence within the TIR domain of MyD88 [83]. Loiarro and co-workers demonstrated that an AntP chimera of this peptide could enter HeLa and HEK293 cells, inhibit MyD88 homodimerization and block IL-1-induced NF-κB [83]. Intriguingly, this group more recently reported that a synthetic peptidomimetic compound (ST2825) modeled on the same BB sequence of MyD88 also blocks IL-1-induced MyD88 homodimerization, IRAK1 and IRAK4 recruitment and NF-κB activation in HeLa cells [84]. Remarkably, oral administration of ST2825 significantly decreased serum levels of IL-6 in mice injected intraperitoneally with IL-1. These findings therefore support the concept that targeting specific signaling events by CPP-Is provides the rationale for the development of further generations of potentially important therapeutic compounds.

Another group of IKK-proximal CPP-Is that block NF-κB activation targets the signaling intermediate TRAF6. TRAF6 functions in multiple pathways including those induced by RANK-1 and CD40 and CPP-Is that block its association with these receptors have been reported [85, 86]. The first of these peptides was derived from the TRAF6 interacting motif in RANK-1 fused with MTS and was named the TRAF6 decoy peptide (L-T6DP-1) [86]. L-T6DP-1 blocked RANK ligand (RANKL)-induced NF-κB activation in macrophages whereas an MTS-fusion peptide encompassing the similar region in RANK-2 (L-T6DP-2) did not. This is consistent with the lower affinity of TRAF6 for RANK-2 and confirms the specificity of the effects of these separate CPP-Is. Further studies with L-T6DP-1 verified the importance of the RANK-TRAF6 interaction for a number of RANKL-induced responses including osteoclastogenesis *in vitro* [86], HIV induction in PBMCs [87] and growth inhibition of monocytes [88]. Using a similar approach Mukundan et al. [85] demonstrated that a CPP-I consisting of MTS and the TRAF6 interaction domain of CD40 blocks CD40L-induced IL-1β, TNF and IL-6 expression in elutriated primary human monocytes. In contrast this peptide did not inhibit PMA-induced pro-inflammatory cytokine expression in monocytes: a response that bypasses the requirement for TRAF6.

Although each of these IKK-proximal CPP-Is was shown to block NF-κB activation, none were absolutely specific for the NF-κB pathway. This is not surprising as these peptides were developed to target signaling intermediates that function upstream of multiple mechanisms. Consistent with this, these CPP-Is inhibit other receptor-induced signals including those activating PKR, JNK and ERK1/2. Nevertheless, these reagents have allowed investigators to directly interrogate the importance of the molecular interactions that these peptides target. As a result, the demonstrated *in vivo* efficacy of some of the CPP-Is confirms the therapeutic relevance of disrupting these crucial signaling mechanisms. These CPP-Is have also revealed important, and in some cases unexpected, molecular insight into the signaling events that they disrupt making them valuable new weapons in the arsenal of target-specific inhibitors available for signaling research.

We will focus the remainder of our discussion on the CPP-Is specifically designed to target key interactions or signaling events at, or downstream of, the IKK complex. These inhibitors and their targets are described in Table 2 and the positions within the classical NF-κB signaling cascade that they target are depicted in Figure 3.

CPP inhibitors of NF-κB nuclear localization

In 1995, Jacek Hawiger and colleagues were first to describe a CPP-I specifically designed to block NF-κB activation [26]. The CPP-I they developed contained the nuclear localization sequence (NLS) of p50 (N50: VQRKRQKLMP) preceded by MTS (the hydrophobic signal peptide CPP) and was termed SN50 for signal peptide:NLS of p50. This peptide functioned by blocking the nuclear translocation of NF-κB. In contrast, a version of the peptide containing a mutated NLS sequence (VQR***NG***QKLMP; mutated residues in bold italics) had no effect. In addition to being the first CPP-I directed against NF-κB signaling, SN50

was also the first intensely utilized CPP-linked inhibitor of any signaling pathway. In this regard, the initial studies of SN50 defined the field of signal-directed CPP-I transduction. In the original manuscript, a ^{125}I labeled SN50 peptide was used to verify its cell permeability [26] providing the first direct evidence of the feasibility of this approach for delivering bioactive cargo targeting the NF-κB pathway. SN50 provided an exciting new reagent to probe the biological relevance of NF-κB activation and it was shown to dose-dependently inhibit LPS- and TNF-induced nuclear translocation of NF-κB in a range of cell types including fibroblasts, monocytes and vascular endothelial cells. Importantly, signal-induced IκBα degradation remained intact in SN50-treated cells, demonstrating that the peptide specifically blocked the nuclear transport of NF-κB [26]. Many later studies have added to the extensive list of stimuli whose responses are blocked by SN50 and the cell types in which it has been effectively utilized (see below).

The mechanism by which SN50 inhibits NF-κB nuclear localization has been resolved. The p50 NLS interacts with a nuclear transport localization complex that includes the importin-α (Rch1/Karyopherin-α2)/importin-β heterodimer [89]. The importin complex exists in the cytoplasm and at nuclear pores and facilitates the entry of the protein being carried through the pore. Typically NLS sequences bind to importin-α, are targeted to the nuclear pore by importin-β, freed from importin-β and translocated into the nucleus with importin-α in an energy-dependent step mediated by Ran GTPase [90]. SN50 prevents this system from transporting NF-κB p50 by competing with the importin complex for p50 [89]. As a result, the importin complex is occupied by SN50 leaving p50 in the cytoplasm.

As SN50 blocks p50 translocation by binding the importin complex, it is not surprising that it was found in later studies to inhibit nuclear entry of other transcription factors that utilize importin [41, 89]. In particular, PMA/ionomycin- and IFN-γ-induced nuclear translocation of AP-1, NFAT and STAT-1 in Jurkat T cells was shown to be inhibited by SN50 [89]. This blockade resulted in abrogation of IL-2 mRNA synthesis, a process that depends upon AP-1 and NFAT in addition to NF-κB. Mutant SN50 peptides containing an altered NLS are not able to bind to importin and failed to inhibit the translocation of any of these transcription factors. Separate studies using primary human lymphocytes did show that at low doses, SN50 specifically blocked NF-κB without inhibiting AP-1 and NFAT [91]. However, in light of its effects on multiple transcription factors, SN50 has more recently been defined as an inhibitor of the general stress-responsive transcription factor (STRF) program [92 – 94]. But despite these obvious specificity issues, SN50 remains a widely used reagent in studies of NF-κB function *in vitro* and *in vivo*. Over two hundred peer-reviewed articles have reported the utility of SN50 in this capacity, although clearly its potential effects on other transcriptional mechanisms must be very carefully considered when interpreting the results of these studies [92, 95].

Some of the most intriguing reports of SN50 function have been in the field of neuroscience and include the demonstration that nerve growth factor serves as a sympathetic neuronal survival factor *in vivo* [96]. Similarly, SN50 was used to demonstrate that nerve growth factor can induce apoptosis when it is unable to provide physiologic stimulation of NF-κB activation [97]. *In vivo* SN50 was used to show that dopamine- [98], NMDA receptor- [99], or AMPA receptor-induced [100] apoptosis of rat striatal neurons requires NF-κB function. Thus, SN50 has served as a very useful tool for dissecting the pro- and anti-apoptotic roles of NF-κB in neuronal cells and these accumulated data suggest that an NF-κB targeted CPP-I approach may hold promise in neurodegenerative diseases characterized by excessive neuronal apoptosis.

SN50 has also been used extensively to demonstrate the requirement for dysregulated NF-κB activity in the survival and proliferation of cancer cells. As an example, the peptide induces apoptosis in multiple myeloma (MM) cell lines as well as *ex vivo* MM cells from patients with active disease [101]. Further evidence supporting the efficacy of SN50 *in vivo* comes from studies of LPS-induced endotoxic shock in mice in which intraperitoneal injection of a cyclic version of the peptide (cSN50) effectively blocked pro-inflammatory cytokine production and lethality [94]. In addition, cSN50 prevented liver apoptosis and hemorrhagic necrosis in LPS-treated mice that may be due in part to the inhibition of expression of pro-apoptotic proteins such as Bax [93]. While these findings suggest that inhibition of NF-κB is critical for the observed effects, it is more likely that the overall efficacy of cSN50 is due to its ability to block multiple SRTFs. Nevertheless these studies add strong support to the potential therapeutic application of CPP-Is based on the p50 NLS for directly inhibiting nuclear translocation *in vivo*.

Since the discovery of SN50, at least five other CPP-Is directed against NLSs have been developed and used to block NF-κB nuclear translocation. The first of these use the same CPP and NLS sequences, but are ligated via a thiazidolino linkage [41]. These CPP-Is, termed ScN50A and ScN50B, worked at least as well as SN50 in blocking NF-κB translocation in PMA/ionomycin-stimulated Jukat cells but similar to SN50

they also blocked the translocation of NFAT and AP-1. Another CPP-I targeting the p50 NLS uses AntP in place of MTS as the transduction sequence. This peptide is termed PN50 (for Penetratin NLS of p50) and has been shown to reduce TNF-induced NF-κB activity in fibroblasts. *In vivo*, PN50 reduces NF-κB activation and improves pancreatitis induced by cholecytstokinin in mice, a model in which NF-κB activity directly correlates with severity of disease [102]. Similar to SN50 it is presumed that PN50 will inhibit the nuclear localization of other transcription factors as it contains the p50 NLS. This, however, has not yet been reported.

The second series of novel NLS-targeting CPP-Is also use MTS, but derive their cargo from c-myc or SV40 [103]. Both of these peptides consist of the MTS flanked on either side by the respective NLS: PKKKRKV for the SV40 construct (named BMS-205820) and AKRVKL for the c-myc construct (named BMS-214572). The reason for using two NLSs was the discovery that a single molecule of importin-α binds 2 NLS sequences [103]. Thus, the dual NLS based CPP-Is were hypothesized to be more potent and were in fact 35-fold more active than SN50 in some experiments [104]. Both BMS-205820 and BMS-214572 prevent LPS induced NF-κB translocation and IL-6 production and encouragingly, these peptides are more specific for NF-κB than SN50, as they do not affect the nuclear translocation of NFAT under identical conditions. BMS-205820 has also been tested *in vivo* and found to significantly enhance survival in murine LPS-induced shock, and it reduces the severity of disease in murine DSS-induced inflammatory bowel disease [104].

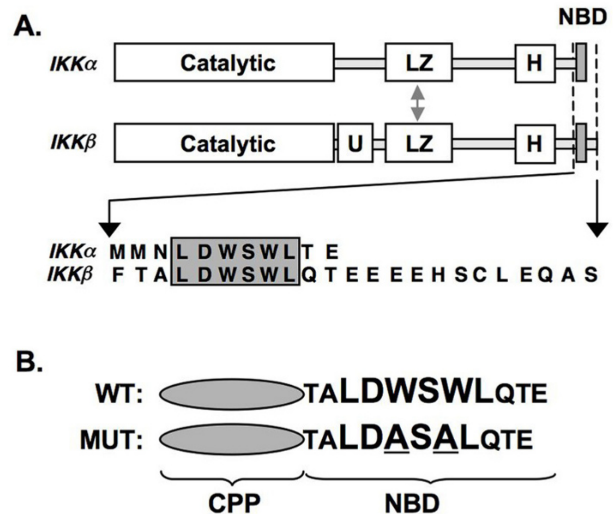

Figure 4. The NEMO binding domain and the NBD peptide. (*A*) The catalytic domains, leucine-zippers (L) and helix-loop-helix (H) of IKKα and IKKβ are shown and a ubiquitin-like domain present only in IKKβ is indicated (U). The position and sequence of the NBD (N) in each kinase is expanded and shown below. (*B*) Sequences of the wild-type (WT) and mutant (MUT) NBD peptides. The CPP is indicated (oval) and the NBD sequence is shown in upper case. The positions of the alanine-substituted tryptophan residues in the MUT peptide are underlined. CPPs that have been used to deliver the NBD peptide include AntP, TAT and PTD-5 [25, 64, 111, 113].

The NEMO binding domain (NBD) peptide

The central role of the IKK complex for NF-κB signaling makes it an attractive target for the development of NF-κB-specific drugs or blocking reagents. While most pharmaceutical companies focus on developing IKK-specific inhibitory compounds [5,6], the role of NEMO as the critical regulatory subunit has not escaped the attention of researchers seeking to manipulate the IKK complex. In this regard, three CPP-Is have been developed that directly target NEMO: the NEMO-binding domain peptide that we will describe here, and more recently, two peptides that block NEMO oligomerization that we will discuss in the following section.

Early studies of the IKK complex suggested that NEMO interacts only with IKKβ [105] and mutational analysis revealed a six amino acid segment (L737 to L742: LDWSWL) within the extreme COOH-terminus of the kinase that was absolutely necessary for NEMO binding (Fig. 4) [64, 65]. More recent studies indicate that a slightly larger region encompassing residues T735 to E745 represents the full domain required for NEMO-association [106 – 108]. This region was named the NEMO binding domain (NBD) and substitution of the tryptophan residues (W739 and W741) with alanine prevented the interaction with NEMO [64, 65]. Remarkably, the identical core of six amino acids are also present in the COOH-terminus of IKKα (Fig. 4) and NEMO in fact associates with IKKα via this functional NBD [64, 65]. Subsequent studies have verified the association of NEMO with both IKKs, although the role of NEMO in regulating the function of IKKα remains somewhat obscure [63, 109].

Initial biochemical analysis using GST pull-down assays demonstrated that a peptide spanning the NBD could block NEMO binding to both IKKs [64]. In contrast, a peptide containing the tryptophan mutations (Fig. 4) failed to block, thereby demonstrating the requirement for the intact domain. Intriguingly, these *in vitro* studies also revealed that the affinity of IKKβ for NEMO was slightly higher than that of IKKα, possibly accounting for the previous failure of others to detect the association with IKKα [105]. To determine whether this NBD spanning region could affect signaling in cells, an AntP

fusion peptide was generated and used to transduce HeLa Cells [64]. This peptide effectively blocked TNF-induced IKK and NF-κB activation but did not affect c-jun phosphorylation, demonstrating both the cellular delivery of the peptide and its specificity for NF-κB signaling.

The AntP version of the NBD peptide and the inactive mutant control (Fig. 4) have been used extensively to block NF-κB in a wide range of cellular and *in vivo* studies. A number of recent reports have also demonstrated the successful transduction of the NBD sequence into several different cell types and *in vivo* using both TAT and PTD-5, demonstrating that the CPP used for delivering the NBD is interchangeable [25, 38, 110 – 114]. Furthermore, recent pharmacological data comparing the NBD peptide delivered by AntP and TAT demonstrated that neither CPP-NBD combination is cytotoxic within their effective dose ranges, although both TAT- and AntP-NBD exhibit significant cytotoxicity at higher doses [37, 38]. In a separate study PTD-5-NBD was also found to be non-toxic and it actually rescued pancreatic islet cells from IL-1β-induced cell death [111]. Thus, regardless of the CPP used to deliver the sequence, doses of the NBD that effectively block NF-κB activation are non-toxic *in vivo* and in the majority of cell types so far examined.

Below we will highlight reports in which the NBD has been applied to cellular studies of NF-κB in inflammation, infection and immunity, cancer, cellular stress and cytoprotection. We will conclude this section by describing how the NBD peptide has been utilized *in vivo* in relevant animals models of disease.

Inflammation

The best characterized NF-κB activation pathways are those elicited by the pro-inflammatory cytokines TNF and IL-1 that function via the classical mechanism [2, 7]. The dependence on NEMO for classical NF-κB-induced gene expression led a number of groups to use the NBD peptide to block cytokine-induced NF-κB in cell culture models including HeLa cells [64, 115]. The first evidence of disease relevant signal inhibition came with the original description of the NBD peptide in which it demonstrated effective blockade of TNF-induced expression of the leukocyte adhesion molecule E-selectin by human umbilical vein endothelial cells [64]. Subsequent studies in endothelial cells also demonstrated that the NBD blocks TNF- and thrombin-induced expression of the store-operated calcium channel TRPC, thereby preventing augmented Ca^{2+} entry and TNF-induced endothelial cell injury [116, 117]. The effect of NBD blockade on IL-1 signaling has more recently been confirmed by Fukashima and colleagues, who demonstrated inhibition of IL-1 induced IκBα degradation in periodontal ligament cells [118]. Furthermore, as part of an *in vivo* study of IL-1-induced brain inflammation Nadjar et al. showed that the NBD peptide blocks IL-1–induced p65 translocation in rat glioma cells [119].

Studies of other pro-inflammatory mediators have further established the ability of the NBD peptide to block NF-κB activation and pro-inflammatory gene expression. In this regard, the NBD was shown recently to inhibit NF-κB and chemokine expression in pancreatic acinar cells incubated with substance P, a neuropeptide that plays an important role in the pathogenesis of acute pancreatitis and certain other inflammatory diseases [120]. The NBD has also been effectively used to block NF-κB induced by the TNF family member RANKL and studies of bone destruction in inflammatory diseases have demonstrated inhibition of RANKL-induced NF-κB activation in osteoclasts [88, 113, 114, 121]. Using the AntP version of NBD, Jimi et al. demonstrated inhibition of RANKL-induced osteoclastogenesis *in vitro* [121] and similar effects were reported by Dai and colleagues using a TAT-NBD peptide [113]. This group also demonstrated that TAT-NBD blocks NF-κB activation in osteoclast progenitors and *in vitro* osteoglastogenesis induced by PMMA particles [114]. Together with the *in vivo* data generated in each of these studies (discussed below), these findings clearly demonstrate that the NBD peptide is an effective blocker of both acute and chronic inflammatory signaling in a wide range of cell types and cellular models of inflammatory disease.

Infection and immunity

NF-κB activation has been intensely studied in the context of infection and immunity, where it functions in both innate immune activation and the development and activation of the adaptive immune response. The recent wealth of knowledge concerning innate immune activation and, in particular, signaling via the TLR family of receptors has led to an upsurge of interest in the role of NF-κB in regulating these events. Due to the crucial role of classical NF-κB in innate immune recognition of bacterial and viral antigens, as well as in dendritic cell activation and lymphocyte development, the NBD peptide has been used in a host of *in vitro* studies of infection, TLR signaling and immune cell activation.

In studies dissecting the role of NF-κB activation in regulating innate immune responses to pathogens, the NBD peptide has been reported to block signaling and

gene expression in cells incubated with the parasites *Theileria parva* and *Trypanosoma cruzi* [122, 123], the bacteria *Escherichia coli*, *Streptococcus pneumoniae, Legionella pneumophila* and *Moraxella catarrhalis,* [124 – 128], and the single stranded negative sense RNA virus of the *Paramyxoviridae* family, *Respiratory syncytial virus* (RSV) [129]. In addition, the NBD peptide inhibited NF-κB-dependent gene expression in cells stimulated with bacterial toxins including vacuolating cytotoxin (VacA) from *Helicobacter pylori* [130] and *Clostridium difficile* toxin A [131]. The ability of the peptide to inhibit TLR signaling was first demonstrated in macrophages in which the NBD blocked LPS-induced nitric oxide production most likely via inhibition of iNOS expression [64]. Subsequent work from Choi et al. showed that TAT-NBD completely inhibited LPS-stimulated IKK activation, NF-κB activity and gene expression in neutrophils [110]. Separate studies of TLR signaling have confirmed that the NBD peptide effectively blocks innate-immune NF-κB activation and responses including chemokine expression [132, 133], upregulation of TLR2 surface expression [134], induction of anti-apoptotic genes [135], upregulation of integrin expression [136] and induction of the intestinal epithelial cell-derived cytokine thymic stromal lymphopoietin (TSLP) [137].

A number of studies have utilized the NBD peptide to examine adaptive immune cell activation and it has been reported to be effective in several models of T cell activation and development. Incubation of T cells with the peptide inhibits TCR-induced NF-κB activation [138, 139] and blocks induction of the trans-activating IκB-like protein Bcl3 which, in turn, affects the generation of Th1 effector T cells [140]. Two studies have also demonstrated inhibition of anti-apoptotic gene expression in T cells and enhanced cell death in response to TLR [135] or TCR [138] stimulation supporting a key role of IKK/NF-κB signaling in lymphocyte development. Elegant *ex vivo* differentiation studies by Igarashi et al. further demonstrated that the NBD peptide blocks B and NK cell development from bone marrow cells in culture by preventing their ability to resist TNF-mediated inhibitory effects [141]. Separately, the peptide was reported to enhance apoptosis in B cells and block the protective effects of BCR-activated Bcl10 on B cell survival [142]. Intriguingly, inhibition of NF-κB in dendritic cells (DCs) by the NBD peptide blocks the expression of IL-6, IL-12 and TNF and significantly affects T cell proliferation and Th1/Th2 polarization in allogeneic mixed lymphocyte cultures [143]. Additional studies in which DCs were activated following cytokine withdrawal by cross-linking cell surface B7-DC with a specific IgM antibody demonstrated that cell survival was dependent upon NF-κB as treatment with the NBD peptide led to enhanced apoptosis [144]. These studies therefore identify the NBD peptide as a powerful tool for the manipulation of the IKK complex in DCs, T and B cells *in vitro*. As we will discuss below, evidence for the efficacy of the NBD peptide in blocking adaptive immune responses *in vivo* in models of chronic immune-mediated inflammation is also beginning to emerge [145].

Cancer

A number of *in vitro* studies utilizing the NBD peptide have focused on its ability to block aberrant NF-κB activity in a range of cancer cell lines. The role of constitutively active NF-κB in certain types of cancer cells has been the subject of intense scrutiny over the last few years since it was found to regulate anti-apoptotic and cell survival genes that rescue cancer cells from exogenous apoptotic stimuli. This allows these tumor cells to survive and proliferate and it is now well recognized that inhibition of NF-κB renders such cells sensitive to apoptotic cell death [4, 146]. Most of these inhibition studies have employed non-selective inhibitors of NF-κB; the NBD peptide, however, has verified the role of NEMO-dependent IKK activity in some tumor cells and has been shown to effectively block dysregulated NF-κB in a variety of cell-based models of cancer.

The effects of the NBD peptide on tumor cells were first reported in the human breast carcinoma cell line MCF-7 that is rendered susceptible to TRAIL- or TNF-induced apoptosis following NBD treatment [147 – 149]. Similar studies of pancreatic and colon adenocarcinoma cells also demonstrated sensitization to TRAIL [149, 150] and the NBD was reported to block proliferation and induce apoptosis in human breast cancer cell specimens [151, 152]. In addition to rendering tumor cells susceptible to exogenously applied apoptotic stimuli, the NBD peptide also inhibits the basal proliferation and survival of several tumor cells including multiple myeloma [153], Hodgkin's lymphoma [154], pancreatic cancer [155] and head and neck squamous cell carcinoma [156]. In each of these studies, the effects of the NBD peptide have been correlated with inhibition of NF-κB activity and subsequent reduced proliferation, increased cell death or downregulation of anti-apoptotic and pro-survival gene expression.

NF-κB also plays a critical role in the resistance of some tumors to anti-cancer drugs that induce anti-apoptotic genes and promote cell survival instead of killing the target tumor cells [4]. The NBD peptide has been used in this regard to block drug-induced NF-κB

activation and has been shown to inhibit IKK and NF-κB activation by the microtubule depolymerizing agent nocodazole [157]. This blockade led to enhanced apoptosis in the nocodazole-treated cells. Some genotoxic anti-cancer drugs such as the topoisomerase II inhibitor etoposide (VP16) also activate NF-κB, leading to cancer cell survival and, in a recent study by Shigeki Miyamoto's group, the NBD peptide inhibited VP16-induced NF-κB activation [158]. However, not all NF-κB activation by anti-cancer drugs leads to cell survival and the cytotoxic effects of the antibiotic doxorubicin and its analogues are thought to depend upon NF-κB activation. This cytotoxic role for NF-κB was supported by recent evidence that the NBD peptide suppressed doxorubicin-induced cytotoxicity in myeloid and lymphoid tumor cells [159]. It is clear therefore that the NBD peptide is a useful reagent for experimentally dissecting the precise effects of anticancer drugs on tumor cells and it may also represent a realistic candidate for adjunct therapy aimed at improving the efficacy of some of these drugs.

Cell stress, cytoprotection and cell survival

In addition to cancer cell survival, NF-κB activity also plays a pivotal role in the cytoprotection and survival of normal cells in the face of a wide variety of cellular stresses. In this regard the NBD peptide has been shown to block NF-κB-dependent cell survival in a range of cell types exposed to distinct cellular stresses. For example the stress-induced IL-6 family cytokine cardiotrophin-1 (CT-1) that is released during hypoxia, activates NF-κB in cardiac myocytes and promotes their survival. The NBD peptide blocks CT-1-induced survival demonstrating the key role of NF-κB in this cytoprotective response [160]. The NBD peptide also causes apoptosis in activated hepatic stellate cells (HSC) supporting a role for NF-κB activation in HSC proliferation and pro-fibrotic function following liver injury [161]. In a study of the effects of ethanol on rat gastric mucosal epithelial cells, the NBD was shown recently to block ethanol-induced NF-κB and COX-2 expression and enhance apoptosis [162]. Finally, studies in neutrophils have revealed that TAT-NBD blocks TNF-induced cell survival after heat injury, again demonstrating a pro-survival role for NF-κB in response to exogenous stress [112].

in vivo studies of the NBD peptide

The NBD peptide was originally shown to be effective *in vivo* in two models of acute inflammation [64]. The first of these was a mouse model of zymosan-induced peritonitis hallmarked by rapidly enhanced neutrophil infiltration and increased peritoneal exudate volume. In this model, intraperitoneal injection of the NBD peptide blocked inflammation by inhibiting both of these parameters. In the same study topical application of the peptide blocked PMA-induced ear edema, again demonstrating the ability of the peptide to inhibit local acute inflammatory reactions. These two experiments not only established the *in vivo* efficacy of the NBD peptide but they also demonstrated its ability to be successfully delivered via separate routes of administration (i. p. and topical). Following this initial description, the anti-inflammatory activity of the NBD was further established in mice in a carrageenan-induced paw edema model of acute inflammation [163]. In this study, the wild type peptide effectively reduced footpad swelling whereas the mutant had no effect. Biochemical and molecular analysis further revealed inhibition of NF-κB and significant reduction of COX-2 and TNF expression in paw tissue from the animals injected with wild-type NBD [163]. In a study of cerulein-induced acute pancreatitis, Ethridge et al. demonstrated that i. p. injection of the NBD ameliorated the resulting inflammation by reducing edema, hemorrhage and neutrophil influx [164]. Three recent reports have also demonstrated the effectiveness of the NBD peptide in blocking inflammation-induced bowel injury [165, 166]. In the first of these studies, De Plaen et al. investigated the role of NF-κB in regulating the profound intestinal injury observed in a rat model of neonatal necrotizing enterocolitis (NEC) [165]. Consistent with a major role for aberrant NF-κB signaling in NEC, the wild-type but not mutant NBD peptide decreased mortality and significantly ameliorated bowel injury in this model [165]. In the second study Shibata and colleagues employed two mouse models of colitis (dextran sulfate sodium salt [DSS] in the drinking water and trinitrobenzene sulfonic acid enema) that are hallmarked by increased NF-κB activity and pro-inflammatory cytokine expression in inflamed intestinal tissue [166]. In both of these models the NBD peptide inhibited NF-κB activation, blocked increased cytokine expression and prevented inflammatory injury. In the third model, Dave et al. explored the effects of the NBD peptide in a model of spontaneous chronic murine colitis in mice lacking IL-10 [167]. Using a version of the NBD fused with 8K as the CPP these workers demonstrated that 8K-NBD ameliorated colitis, inhibited NF-κB activation in the

lamina propria, and blocked expression of pro-inflammatory cytokines in the intestine.

The NBD peptide has also been applied to investigations of the pathological role of NF-κB in lung inflammation. Haeberle and colleagues demonstrated that intra-nasal administration of the peptide inhibits acute inflammation in the lungs of mice infected with RSV [129]. In this study the peptide inhibited NF-κB activity in infected lungs and ablated RSV-induced chemokine expression and inflammation-associated pathology. More recently, Chapoval et al. showed that the peptide reduced IL-13-induced tissue inflammation, fibrosis and alveolar remodeling in an IL-13-transgenic mouse model of asthma [168]. In separate studies, topical nasal administration of the NBD peptide in piglets inhibited severe inflammation induced by repeated airway lavage [169, 170]. In this model of acute respiratory distress syndrome the peptide significantly reduced lung edema, protein content in the epithelial lining fluid, PMN accumulation and leukotriene B4 levels and improved the functional residual capacity, alveolar volume and lung mechanics [169, 170]. Importantly, in addition to verifying the ability of the NBD peptide to ameliorate lung inflammation, these studies are the first to establish its efficacy in large animal models of disease.

Consistent with the ability of CPPs to deliver functional protein and peptide cargos across the blood-brain barrier [48 – 50, 171], the NBD-peptide enters the brain and inhibits NF-κB in models of inflammation and injury. In this regard, Nadjar and colleagues have established that intraperitoneal as well as intracranial injection of the NBD peptide effectively blocks IL-1-induced NF-κB activation and COX2 expression in brain microvascular endothelial cells and ameliorates sickness behavior in rats [119, 172]. In a separate study, it was also shown that intraperitoneal injection of the NBD peptide up to 12 hours after reoxygenation following hypoxia/ischemia (HI)-induced cerebral damage inhibited NF-κB activity in rat brain tissue [173]. Intriguingly however, NF-κB inhibition after reoxygenation enhanced the brain damage observed six weeks after HI [173]. More recent studies have demonstrated that the timing of administration of the NBD peptide in this model is absolutely critical and that treatment of rats either immediately or up to six hours after HI leads to almost complete inhibition of the brain damage observed six weeks later [174, 175]. The NBD peptide has also shown promising efficacy in a mouse model of Parkinsons disease (PD) in which it prevented nigrostriatal degeneration and dopaminergic neuron loss [176]. In this model, intraperitoneal injection of the peptide inhibited NF-κB activation and blocked pro-inflammatory cytokine expression in the brains of experimental mice. Moreover, NBD-treated mice exhibited improved activity levels and motor functions, strongly suggesting that NF-κB-induced inflammation contributes to PD and is thereby a viable therapeutic target.

The ability of the NBD peptide to inhibit chronic inflammation has also been demonstrated. In two separate reports, the peptide inhibited collagen-induced arthritis in mice by blocking *in vivo* NF-κB activation in osteoclasts, resulting in reduced osteoclastogenesis and focal bone erosion [113, 121]. Systemic application of the peptide in these studies also reduced lymphocyte infiltration and chronic inflammatory pathology in the joints. Similar inhibition of synovial inflammation by the peptide was also demonstrated in a model of adjuvant arthritis in rats [177]. Further evidence for the efficacy of the NBD against inflammation-induced bone loss emerged from a study of osteoclastogenesis and calvarial inflammatory osteolysis in response to poly(methyl methacrylate) (PMMA) particles [114]. In this murine model of implant failure at the bone implant interface, subperiosteal injection of the TAT-NBD peptide over the calvaria abrogated PMMA-induced inflammation and osteolysis.

The NBD peptide has been studied in the context of chronic inflammation of the CNS in an adoptive transfer model of experimental allergic encephalomyelitis. In this model the clinical symptoms of EAE were significantly reduced in wild-type NBD injected (i. p. / alternate days for >50 days) compared with mutant-injected or control animals [145]. This reduction of symptoms was accompanied by reduced NF-κB activity, a shift in the immune response from a Th1 to a Th2 profile and inhibition of the MBP-specific T cell function. Furthermore, the NBD inhibited the induction of iNOS, IL-1β and TNF in the cerebellum of the EAE animals. In a recent study, the peptide was also shown to block muscle degeneration in the *mdx* mouse model of Duchenne muscular dystrophy (DMD) [178]. These animals develop clinical symptoms similar to DMD patients including elevated pro-inflammatory cytokine levels in their muscles, persistent inflammation hallmarked by immune cell invasion, and degeneration of the diaphragm leading to early lethality. The wild-type NBD inhibited elevated NF-κB activity in the muscles of *mdx* mice resulting in significantly reduced inflammation, muscle regeneration and improved diaphragm function [178].

Taken together these separate *in vivo* studies clearly demonstrate that the NBD peptide is effective in established models of both acute and chronic inflammation. In many of the published models, inhibition of inflammation has been correlated with the inhibition of NF-κB in tissue and the subsequent reduction of

NF-κB-dependent pro-inflammatory gene expression. A number of routes of administration including topical, i. p., intranasal and intracranial, have been tested and the ability of the peptide to function in each of these studies clearly demonstrates that its effects are systemic and that it can reach sites both local and distant from the original point of injection. Furthermore, in some of the studies of chronic inflammation, the NBD peptide was administered for over 50 days on each alternate day without any observable toxicity, liver damage or other detrimental side effects [121, 145]. These acute and chronic inflammation studies were performed using doses of the peptides ranging from 0.75 to 20 mg / kg and, in our experience, these doses are completely tolerated by mice and rats without any observable toxicity. Evidence of toxicity and rarely also of seizure is only observed in mice when doses of over 50 mg / kg are used (unpublished observations); these effects, however, are related to the high concentration of the CPP as similar toxicity is observed with the inactive mutant control peptide or the CPPs alone.

Future applications

Due to the general lack of specificity of the NLS CPP-Is for NF-κB, the NBD peptide is currently the best-studied highly selective cell-penetrating peptide inhibitor of classical NF-κB signaling. As we have discussed, this CPP-I effectively inhibits NF-κB in a wide range of cell models and more importantly, it functions *in vivo* in relevant animal models of disease. Given the number of *in vitro* studies focused on NF-κB in cancer that have utilized the NBD, it is surprising that it has not yet been applied to any *in vivo* models of cancer. The efficacy of the peptide in animal models of acute and chronic inflammation makes this an eagerly anticipated prospect.

One outstanding question regarding the function of the NBD peptide concerns its effects on IKKα. Current dogma dictates that IKKα plays no role in classical NF-κB signaling that requires only NEMO and IKKβ [2, 7 – 9]. Furthermore, NEMO is not thought to function in the non-canonical NF-κB pathway that requires only IKKα. Nevertheless, it is clear that IKKα contains a functional NBD and that the NBD peptide disrupts its interaction with NEMO [63 – 65, 109]. There is currently no known role for NEMO in regulating mammalian IKKα; our recent findings, however, suggest that a NEMO-IKKα complex is sufficient to transduce IL-1- but not TNF-induced signaling to NF-κB [63]. Moreover, a recent report demonstrated that IKKα in zebrafish is regulated via interaction with NEMO [109]. Intriguingly this occurs through an NBD in zebrafish IKKα that contains a very similar "core" to the mammalian domain (QDWSWT compared with LDWSWL in mammals). In the zebrafish system the NEMO-IKKα interaction plays a role in downregulating classical NF-κB activation [109] and it is fascinating to consider such a model for the mammalian IKK complex. Recent evidence from the laboratories of Michael Karin and Inder Verma supports a negative regulatory role for IKKα in the classical pathway [179, 180] and, consistent with NEMO functioning in this negative regulation, we have previously reported that the NBD peptide increases basal NF-κB activity in HeLa and vascular endothelial cells [64]. Nevertheless, further study is required to verify this role for NEMO in basal IKK activity and we predict that the NBD peptide will be a crucial experimental tool for probing the functional relationship between IKKα and NEMO. It is clear therefore that a better understanding of the effects of the NBD peptide on both IKKβ and IKKα function *in vitro* and *in vivo* is required. Indeed, such insight will be crucial if the NBD is to be considered as a potential target for the development of clinically acceptable drugs.

Other CPP inhibitors of NF- κB

CPP-Is targeting the NLS and the NBD are by far the best characterized of the current peptides specifically directed against the NF-κB pathway. However, a number of recent studies have reported the use of peptide transduction to generate reagents that selectively target other critical components of the classical pathway. These novel CPP-Is include two peptides that block NEMO oligomerization and a group of inhibitors that specifically interfere with the phosphorylation of p65 and IκBα (Table 2). Although less is known about the range of effects of these CPP-Is, they have each been shown to block signal-induced NF-κB activation and its functional activity in cells and in some cases *in vivo*. It is therefore likely that these inhibitors will become more widely used as researchers seek to selectively target NF-κB in animal models of disease.

Inhibitors of NEMO oligomerization

NEMO oligomerization is absolutely critical for signal-induced IKK complex kinase activity [24, 68 – 71] and two novel CPP-Is that disrupt this process were described recently [68]. Oligomerization requires both the second coiled-coil (CC2) and leucine zipper (LZ) domains of NEMO (Fig. 5) and together

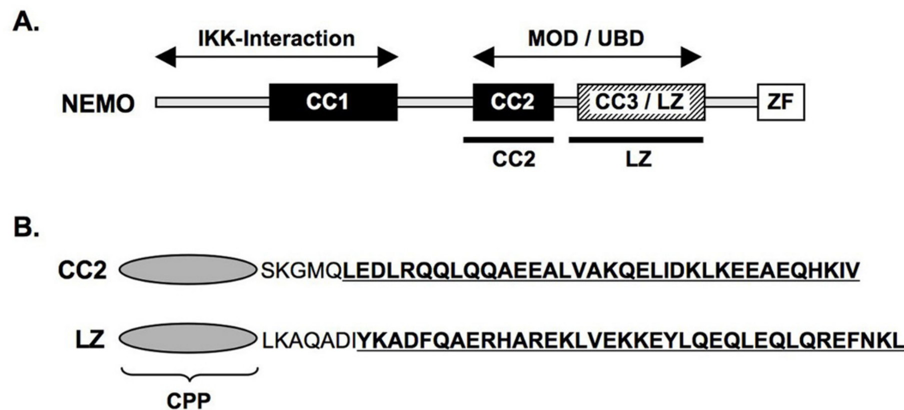

Figure 5. CPP-Is targeting NEMO oligomerization. (*A*) The first and second coiled-coil domains of NEMO are shown in black (CC1 and CC2) and the third coiled-coil that overlaps a leucine zipper region (CC3/LZ) is hatched. The COOH-terminal zinc-finger motif (ZF) and the NH_2-terminal region required for interaction with the NBD in the IKKs are indicated. Shown also is the region encompassing the CC2 and CC3/LZ domains that constitutes the minimal oligomerization domain (MOD) / ubiquitin binding domain (UBD). The positions of the CC2 and LZ regions used for generating the two CPP-Is are drawn (lines) (*B*) Sequences of the CC2 and LZ peptides. The CPP is indicated (oval) and the protein-derived sequences are shown. The residues in bold and underlined are those encompassing the complete CC2 and LZ domains. CPPs that have been used to deliver these peptides include AntP and R7 [24, 68].

with the intervening linking sequence, these are named the minimal oligomerization domain (MOD). To target the MOD in mouse NEMO, Agou and colleagues designed two AntP-chimeric peptides directed against each of these regions. The first peptide consisted of the full length of CC2 (named AntP-NEMO-CC2) and the second encompassed the complete LZ domain (named AntP-NEMO-LZ) (Table 2; Fig. 5) [68]. Biophysical analysis demonstrated that both of these peptides could bind to NEMO and functional assays verified that they disrupted its oligomerization *in vitro*. Importantly, the ability of AntP-NEMO-CC2 and –LZ to block NF-κB activity was demonstrated in LPS-stimulated 70Z/3 mouse pre-B cells whereas neither peptide inhibited p38 or ERK signaling. Agou and coworkers also generated control peptides by selectively substituting critical resides in the CC2 and LZ domains and none of these mutants disrupted NEMO oligomerization or blocked NF-κB activation in cells [68].

Despite these extremely promising results, one issue concerning the precise mode of function of these CPP-Is remains to be resolved. Recent studies have reported that the ability of NEMO to interact with upstream K63-linked polyubiquitin chains relies upon the ubiquitin-binding domain (UBD) that overlaps with the MOD [66, 67] (Fig. 5). Since ubiquitin-binding by NEMO is required for the induced activation of the IKK complex, it is possible that either or both of these novel CPP-Is disrupt this process. Further work is therefore required to determine precisely whether NEMO oligomerization or ubiquitin binding (or both processes) is the critical target of the CC2 or LZ peptides. Nevertheless, it remains clear that these peptides effectively block signal-induced NF-κB activity in cells and, collectively, the studies of Agou et al. have firmly established both the target-binding specificity and pathway-selectivity of these novel CPP-Is targeting the MOD/UBD domain.

Functional studies of AntP-NEMO-CC2 and –LZ demonstrated that both peptides significantly enhanced the apoptosis of a retinoblastoma cell line that requires constitutive NF-κB activity for survival [68]. This finding was further supported by the recent demonstration that AntP-NEMO-LZ induced cell death in a panel of human myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) cell lines as well as in bone marrow cells derived from high-risk MDS and AML patients [24]. Intriguingly this latter study was performed using a version of the NEMO-LZ peptide fused with a poly-arginine-based CPP (R7: RRRRRRRLKAQA) in place of AntP, thereby demonstrating the interchangeability of separate CPPs to effectively deliver the same cargo. Taken together therefore, these initial cell-based biological studies of the CC2 and LZ peptides provide convincing proof of principle for this approach and raise the exciting prospect of future studies using these CPP-Is to target the MOD/UBD domain of NEMO *in vivo*.

Inhibitors of p65 phosphorylation

The first CPP-Is specifically developed to interfere with the phosphorylation of NF-κB proteins target the prototypic classical p65 subunit. Serine phosphorylations at positions 276, 529 and 536 have been shown to

be required for the normal transactivating function of p65 [54, 55, 181] and armed with this understanding Takada and colleagues designed a panel of peptides targeting these residues [182]. To target S276, a peptide including this residue (bold and underlined: [271]QLRRP**S**DRELSE[282]) was fused with AntP and this CPP-I was named AntP-p65-P1. A second CPP-I named AntP-p65-P6 was also generated that encompassed both S529 and S536 ([525]NGLL**S**GDEDF**S**S[537]). Both of these CPP-Is effectively blocked TNF-, LPS- and IL-1-induced NF-κB activation in KBM-5 chronic myeloid leukemia cells whereas neither prevented the phosphorylation or degradation of IκBα or TNF-induced AP-1 activation. Instead, these peptides prevented the phosphorylation of p65 and blocked its nuclear translocation and transcriptional activity. Furthermore, AntP-p65-P1 was shown to inhibit NF-κB-dependent gene expression (i. e. CyclinD1, COX2 and MMP9). Importantly replacing S276 with alanine abrogated the inhibitory activity of the peptide thereby providing both target verification and a convenient control peptide for experiments utilizing this reagent. The biological activity of AntP-p65-P1 was established in studies of human head and neck squamous cell carcinoma cells in which it blocked both constitutive NF-κB activity and cell proliferation [156]. Furthermore, AntP-p65-1 rendered KBM-5 cells susceptible to TNF-induced apoptosis, once again highlighting both the critical role of NF-κB in tumor cell proliferation and survival and the ability of target-specific CPP-Is to block this pathophysiological function of NF-κB [156, 182]. These CPP studies therefore identify p65 phosphorylation as a realistic target for the development of NF-κB-specific drugs and provide valuable insight supporting further efforts aimed at using CPPs to directly target post-translational modifications of the NF-κB proteins.

CPP-Is targeting IkB*α*:

Another group of CPP-Is that inhibit NF-κB activation by blocking a critical phosphorylation event does so by specifically targeting IκBα. Since the phosphorylation of Serines 32 and 36 is required for the subsequent ubiquitination and degradation of IκBα in the classical pathway, these CPP-Is target this region of the protein. Notably, each of these CPP-Is consists of a CPP fused with a whole (or truncated) IκBα protein and, as such, these are the only examples of whole protein or large polypeptide transduction approaches currently directed at the NF-κB pathway. This group of CPP-IκBα chimeras was constructed by subcloning the IκBα cDNA into expression vectors harboring the appropriate CPP sequence together with an epitope tag (e.g. His, HA or GST). The CPP-I fusion proteins were then expressed and biochemically purified using the convenient tags and then used to transduce target cells or inject *in vivo*.

The original IκBα-specific CPP-Is consisted of the full-length IκBα protein in which S32 and 36 are substituted with alanine that was preceded at the NH_2-terminus by TAT [183, 184]. This construct is named the TAT super-repressor or TAT-srIκBα and it has been used to block IL-1β- and TNF-induced NF-κB activation in HeLa, A459 and Jurkat cells without affecting ERK, JNK or p38 MAP kinase signaling [183, 184]. TAT-srIκBα also renders eosinophils more susceptible to TNF-induced apoptosis [183] and it has been used *in vivo* to block carrageenan-induced pleurisy in rats following intravenous injection [185]. A second CPP-I that targets IκBα consists of amino acids 37 – 317 (i. e. the whole protein minus the NH_2-terminal phosphorylation domain) fused with MTS as the CPP at the COOH-terminus [186]. This MTS-IκBα(ΔN) construct inhibited NF-κB activation in NIH-3T3 cells and primary thymocytes and functioned *in vivo* to block NF-κB activity in mouse skin after wounding. Intravenous injection of MTS-Iκ Bα(ΔN) also inhibited endotoxin-induced NF-κB activation and the resulting change in pulmonary vascular resistance in sheep lungs thereby demonstrating the effectiveness of this CPP-I in large mammal models [186].

The future of CPPs in NF-κB research

The studies described above clearly demonstrate that CPP-mediated transduction can be effectively used to inhibit classical NF-κB activation induced by a wide range of stimuli in many different cell types. Most exciting however is the ability of CPP-Is targeting NF-κB to function *in vivo* in established models of disease. The full potential of the NF-κB CPP-Is that have been developed has not yet been realized and we predict that these reagents will be used increasingly frequently by researchers wishing to selectively inhibit NF-κB signaling at well-defined molecular targets. Furthermore, as we better understand the unique signaling components that function in the myriad of pathways leading to the many NF-κB transcriptional responses, the future use of CPPs to target these will most likely proliferate. We therefore anticipate that more CPP-Is targeting NF-κB activation will emerge and that these will disrupt novel interactions or signaling events within the many pathways that are currently under investigation.

Expansion of our knowledge of NF-κB signaling will be accompanied by greater insight into the mecha-

nisms, capabilities and limitations of peptide transduction technology. In particular, the precise mechanisms of cellular entry and intracellular release of each of the classes of CPPs will be characterized. This understanding will permit the design of new generations of synthetic CPPs with improved transduction, cargo release and target accessing capabilities. New classes of CPPs will also be identified and used to deliver NF-κB-directed cargos. In this regard, small pentapeptide CPPs (named CPP5s) derived from a Bax (Bcl2-associated X protein)-binding partner were identified recently and shown to rapidly enter many cell types in an endocytosis- and pinocytosis-independent manner [187]. CPP5s exhibited very low toxicity in cells and *in vivo* and it is therefore likely that these will be tested in the near future for their ability to deliver cargos directed at the NF-κB signaling pathways.

Advancement will also be made in determining the pharmacokinetics of the separate classes of CPPs and, more importantly, the CPP-Is targeting NF-κB. TAT has been shown to deliver bioactive cargo to all tissues in mice [42, 43] and in a recent study, Cai and colleagues described the tissue distribution kinetics of a TAT fusion with β-galactosidase following oral, i. v., i. p and portal vein administration [44]. Although this was not a definitive pharmacokinetic study, the encouraging results provide the basis for future in-depth studies and lend strong support to the use of TAT in developing peptide transduction-based therapies. Accumulated evidence from *in vivo* studies of the NBD peptide demonstrates its efficacy in models of diseases in a wide array of distinct tissues. Furthermore, the NBD has been administered via numerous routes including i. p., intranasal, intracranial and topical, and it has proven to be effective in each case [64, 121, 129, 163, 167, 168, 172 – 176]. This therefore suggests that this CPP-I has good absorption and tissue distribution kinetics; the precise details of these critical pharmacokinetic parameters, however, remain to be definitively determined. Furthermore, the metabolism and / or excretion of the NF-κB-targeting CPP-Is have not been reported and the potential for detrimental immunogenicity of these peptides has not yet been addressed. Finally, although the ability of each of the CPPs to traverse the blood-brain barrier and enter the CNS may provide the exciting capability of targeting bioactive cargo to these tissues [145, 172, 174 – 176], the possibility of neurotoxicity must be very carefully considered when pursuing these approaches. Clearly, obtaining a detailed understanding of the pharmacokinetics of the individual CPP-Is should be an area of intense effort in the near future if the ultimate goal of bringing these reagents to the clinic is to be attained.

Recent evidence suggests that the efficacy of the NBD peptide *in vivo* varies among disease models and is dependent upon the CPP with which it is coupled [25]. A better understanding of how the biophysical properties of the CPPs affect their pharmacokinetics will therefore allow for more informed and rational selection of the CPP-cargo combinations to most effectively target NF-κB *in vivo*. One related issue that has yet to be addressed in the peptide transduction field is the ubiquitous ability of CPPs to enter all cell types and tissues *in vivo* [13]. In designing strategies to specifically block aberrant NF-κB signaling at sites of disease it would be extremely beneficial to be able to target the delivery of the CPP-Is to only those tissues. We therefore predict that inroads will be made into identifying methods to selectively target CPPs to diseased tissues such as chronic inflammatory lesions or tumors, indeed Myrberg and colleagues have recently described a tumor-targeting peptide sequence that may facilitate this [188]. One further possibility may be the development of synthetic micelle-based carrier approaches to "cage" the CPP-I, then deliver it directly into the local cellular environment at sites of disease. To do this micelles would need to be engineered to selectively home to disease sites possibly by binding to cell surface proteins only expressed in cells present within diseased tissue (e.g. leukocyte adhesion molecules on endothelial cells at sites of inflammation, tumor-specific antigens).

One of the exciting consequences of targeting NF-κB signaling using CPP-Is is the ability of this relatively straightforward technique to validate potential drug targets in cells and *in vivo*. This "proof-of-principle" that CPP-Is provide might then lead to the further development of new generations of drugs directed against those validated targets. Examples of how this has already occurred include the development of an effective peptidomimetic compound modeled upon the BB loops in the TIR domain of MyD88 that blocks IL-1 and TLR signaling [84]. This work emerged directly from studies of CPP-Is targeting the same domain [83]. Using a separate and insightful approach, Wyler and colleagues recently used the identification of CPP-Is targeting the ubiquitin-binding / MOD of NEMO [68] as the rational basis to generate a panel of designed ankyrin repeat proteins (DARPins) directed against the same domains [189]. These DARPins blocked NEMO oligomerization and prevented TNF-induced NF-κB activation in cells. Approaches such as these may have advantages such as better stability *in vivo,* potentially higher target affinity and lower costs of production over using CPP-Is themselves as drugs. However the well-established ability of CPP-Is to function *in vivo* warrants further

intense investigation of peptide transduction as a means in its own right to deliver NF-κB targeting drugs.

Novel targets for NF-κB-specific CPP-Is

A major challenge facing pharmaceutical companies is the development of highly specific NF-κB inhibitors that block only aberrant signaling and maintain normal physiological responses. The importance of this is highlighted by a growing number of genetic studies in mice demonstrating detrimental effects of tissue-specific deletion of components of the IKK complex [190]. In this regard, two recent reports showed that deleting either NEMO or IKKβ from intestinal epithelial cells leads to severe disruption of immune homeostasis in the gastrointestinal tract and results in chronic inflammation resembling inflammatory bowel disease [137, 191]. Other examples of deleterious effects are the increased development of hepatocellular carcinoma in mice with a targeted deletion of NEMO in hepatocytes [192, 193] and the development of inflammatory skin disease in mice lacking IKKβ in keratinocytes [194]. Contrasting these studies, CNS-restricted deletion of NEMO and IKKβ ameliorated autoimmune encephalomyelitis in mice, thereby demonstrating the key role of the classical NF-κB pathway in promoting the inflammation associated with this disease [195]. Nevertheless, the severe detrimental effects of deleting IKK complex subunits in some cell types *in vivo* cannot be overlooked as they suggest that systemic inhibition of NF-κB signaling might have damaging consequences. Notably however, the majority of pharmacological approaches that have been used to target NF-κB *in vivo* have not resulted in deleterious pathology [6]. This is supported by the increasing number of reports of effective amelioration of inflammatory disease using the NBD peptide (discussed in detail earlier). For example, although genetic deletion of IKKβ and NEMO had profound pro-inflammatory effects in the gut [137, 191], treatment of mice with the NBD peptide prevented inflammation in murine models of colitis and necrotizing enterocolitis [165 – 167]. It is therefore difficult to determine whether genetic deletion of separate IKK complex subunits is an absolute predictor of the likely effects of pharmacologicaly targeting IKK activation. One example of where this does appear to be the case is a recent report by Greten and colleagues who showed that deletion of IKKβ in myeloid cells led to increased endotoxic shock due to enhanced IL-1β processing [196]. These results were recapitulated in wild-type mice following prolonged pharmacologic inhibition of IKKβ highlighting the potential problems associated with long term IKKβ inhibition. Clearly, therefore, any drugs targeting the NF-κB pathway must be used with caution and the more selective a drug can be made for specific upstream signaling pathways to NF-κB, the better that drug will be. The ability of CPP-Is to deliver cargos directed at specific molecular interactions or signaling events provides a convenient method to effectively accomplish this critical pathway selectivity.

One example of how pathway specificity has already been achieved using a CPP-I is the AntP-TIRAP peptide, described by Horng and colleagues, that blocks signaling via TLR4 but does not affect NF-κB activation by TLR9 [81]. This occurs because TIRAP is a TLR4- but not TLR9-specific adaptor protein, thereby highlighting the importance of identifying more pathway-specific targets upstream of NF-κB that can be accessed using CPP-Is. An important target protein that could be potentially exploited in this regard is NEMO. The two CPP-I approaches already targeting NEMO inhibit all classical signaling to NF-κB by blocking critical NEMO functions (i. e. IKK binding and oligomerization respectively). However, mutations within NEMO occur in a panel of inherited human diseases and the effects of these mutations range from either complete loss of function to inhibition of distinct subsets of signaling pathways to NF-κB [197, 198]. For example, certain mutations in the COOH-terminus of NEMO block NF-κB activation in response to TNF but not IL-1 [199]. This is also illustrated by the finding that some of these mutations impair the function of the TNF superfamily receptor CD40 on B cells, whereas many mutations elsewhere in NEMO do not (207). These findings strongly suggest that distinct domains of NEMO are required to respond to separate upstream signaling pathways. It is therefore intriguing to speculate that CPP-Is specifically designed to interfere with individual domains or specific posttranslational modifications within NEMO may be able to differentially block signaling leading to classical NF-κB activation.

Another exciting prospect is the generation of CPP-Is specific for the non-canonical NF-κB pathway. All of the current CPP-Is targeting NF-κB disrupt the classical pathway and none have been tested for effects on non-canonical NF-κB activation. While it is possible that the SN50 peptide may affect the nuclear translocation of non-canonical NF-κB heterodimers (i. e. p52:RelB) and that the NBD peptide may affect the function of IKKα by disrupting the endogenous IKK complex, no evidence for this has yet been provided. We predict therefore that novel peptides specifically disrupting unique components or signaling events in the non-canonical pathway possibly

upstream of IKKα activation (i. e. blocking TRAF3 and / or NIK function) will soon be developed to allow researchers to conveniently dissect these pathways in cells and *in vivo*. In addition to being the effector kinase in the non-canonical pathway, it has become clear that IKKα also functions independently of NEMO and IKKβ in the nucleus [200 – 202]. In this setting, IKKα appears to regulate chromatin structure around NF-κB-inducible gene promoters by phosphorylating histones or other proteins involved in chromatin remodeling and gene transcription [200 – 202]. As the major classes of CPPs can enter the nucleus, it may be possible in the future to specifically target these nuclear functions of IKKα using novel CPP-Is.

The full potential of whole protein CPP-Is for modulating NF-κB signaling has not yet been explored. To date only IκBα has been targeted using CPPs fused with whole proteins [183 – 186]; there appears to be no limitation to the power of this approach, however, to manipulate NF-κB. Proteins that could be transduced might include dominant negative versions of pathway-specific kinases. For example, selective ablation of the classical pathway might be possible using CPP-tagged kinase-dead IKKβ whereas inactive IKKα or NIK might effectively block non-canonical signaling. Furthermore, although all of the current NF-κB specific CPP-cargos function as inhibitors of the pathway, it will be possible using whole protein transduction to deliver active signaling intermediates to selectively induce or upregulate distinct pathways. While whole protein transduction may not be best suited for clinical use, it provides researchers with a very convenient method for assessing the effects of immediate expression of exogenous proteins in target cells.

The exciting potential of *in vivo* delivery

The importance of the ability of CPPs to deliver cargo targeting NF-κB activation *in vivo* cannot be overstated. This capability has allowed basic researchers to translate *in vitro* and cell-based findings into relevant animal models, and the potential for applying CPPs to the study the pathophysiological role of NF-κB in disease is immense. The best-studied NF-κB targeting CPP-I in this regard is the NBD peptide that has been shown to effectively ameliorate disease in multiple *in vivo* models of acute and chronic inflammation [25, 64, 113, 121, 145, 163, 165, 169, 172, 177]. In light of these very promising results it may not be long before clinical trials of drugs aimed at blocking NF-κB signaling at the NBD are underway. Recent studies by major pharmaceutical companies that have precisely defined the structure of the NBD suggest that this exciting prospect may indeed be close at hand [106, 108]. As new pathway-specific CPP-Is are developed, these too will be tested *in vivo* for selective effects on aberrant signaling in disease and this will pave the way for novel therapeutics aimed at these validated targets. It is particularly intriguing to note that CPP-Is targeting NF-κB have been successfully tested in large animal models (i. e. piglets and sheep) [169, 186] as these more closely resemble the human situation. CPPs may therefore bring us one step closer to realizing the long sought after goal of developing pathway-specific and clinically safe NF-κB inhibiting drugs.

Summary

CPP technology provides the NF-κB field with a convenient and well-established method to generate useful experimental tools for dissecting this ever more complex signaling paradigm. As we continue to unravel the plethora of interaction domains and post translational modifications that function in the many pathways leading to NF-κB activation, it is likely that realistic targets for the development of novel blocking peptides, substrate decoys or other domains will arise. The relative ease of generation of CPP-Is aimed at these novel targets will put target validation in cells and *in vivo* into the hands of researchers. To date, several CPP-Is targeting the classical NF-κB pathway have been developed and some of these have been demonstrated to be highly effective *in vivo*. Most notably, the NBD peptide that blocks the association of NEMO with IKKα and IKKβ has been shown to inhibit NF-κB and ameliorate disease in models of acute and chronic inflammation. These findings establish an exciting blueprint for the future development of novel CPP-Is targeting the many separate upstream signaling pathways that activate classical and non-canonical NF-κB. The most exciting aspect of these studies is the very real promise that CPP-Is offer for translating basic research into effective clinical strategies. In the continuing effort to bring NF-κB inhibitors from the bench to the bedside, CPP-Is represent a very attractive, realistic and attainable first step in realizing this fundamental objective.

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