Ubiquitination and Degradation of the Inhibitors of NF- κ B

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The key step in NF- κ B activation is the release of the NF- κ B dimers from their inhibitory proteins, achieved via proteolysis of the I κ Bs. This irreversible signaling step constitutes a commitment to transcriptional activation. The signal is eventually terminated through nuclear expulsion of NF- κ B, the outcome of a negative feedback loop based on I κ B α transcription, synthesis, and I κ B α -dependent nuclear export of NF- κ B (Karin and Ben-Neriah 2000). Here, we review the process of signal-induced I κ B ubiquitination and degradation by comparing the degradation of several I κ Bs and discussing the characteristics of I κ Bs' ubiquitin machinery.

Retrospectively, one of the most remarkable milestones in NF- κ B research was the identification of its cytoplasmic inhibitor I κ B (Inhibitor of NF- κ B), soon after the discovery of the DNA binding activity of the factor (Sen and Baltimore 1986a; Sen and Baltimore 1986b; Baeuerle and Baltimore 1988). It immediately focused the research of NF- κ B activation on the mechanism of liberation of the factor from the inhibitory effects of I κ B. Using a simple detergent treatment of cell extracts, Baeuerle and Baltimore provided the first proof-of-concept for a model entailing the dissociation of the inhibitor as a major step in NF- κ B activation (Baeuerle and Baltimore 1988). Subsequent experiments in cell lines suggested that stimulus-induced I κ B phosphorylation triggered release of associated NF- κ B, which could account for physiological

activation of NF- κ B (Ghosh and Baltimore 1990). Yet, later experiments showed that I κ B phosphorylation was insufficient for NF- κ B activation (Alkalay et al. 1995a; DiDonato et al. 1995), and I κ B degradation must precede NF- κ B activation (Beg et al. 1993; Brown et al. 1993; Mellits et al. 1993; Sun et al. 1993). Baeuerle and colleagues showed that blockade of I κ B degradation prevented NF- κ B activation (Henkel et al. 1993), but elucidation of the mechanism of I κ B proteolysis awaited further studies by Maniatis, Goldberg, Ben-Neriah, Ciechanover, and colleagues who showed that signal-induced ubiquitination and proteasomal degradation of I κ B were required for NF- κ B activation (Palombella et al. 1994; Alkalay et al. 1995b; Chen et al. 1995). These findings implicated for the first time ubiquitin-dependent proteolysis as an integral

Editors: Louis M. Staudt and Michael Karin

Additional Perspectives on NF- κ B available at www.cshperspectives.org

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Cite this article as *Cold Spring Harb Perspect Biol* 2010;2:a000166

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step of signal induced transcriptional activation, yet the specificity basis for I κ B degradation remained to be shown. Sequence homology comparisons and site-directed mutagenesis revealed a conserved amino-terminal sequence containing two serine residues that were found to be phosphorylated after phorbol ester stimulation and are necessary for signal-induced I κ B α ubiquitination and degradation by the proteasome (Brown et al. 1995; Chen et al. 1995). This information was critical for the subsequent molecular characterization of both the I κ B kinase and ubiquitin ligase (E3). Using a set of specific phosphopeptides, Yaron et al. showed that the phosphorylation-based motif (DpSGXXpS) of I κ B α is sufficient for I κ B recognition by components of the ubiquitin-system in vitro (Yaron et al. 1997). Subsequently, the specificity component of the E3, β -TrCP, was identified by mass spectroscopy as a protein that specifically interacts with the I κ B phosphopeptide motif (Yaron et al. 1998). Significantly, a *Drosophila* homolog of β -TrCP, *slimb*, had previously been identified by Jiang and Struhl in a genetic screen as a likely E3 for β -catenin and cubitus interruptus (Ci) (Jiang and Struhl 1998). Several labs (Spencer et al. 1999; Tan et al. 1999; Winston et al. 1999) then showed that β -TrCP is the substrate binding (receptor) subunit of an SCF-type E3 ligase (Deshaies 1999). The structural basis for recognition of phospho-I κ B α by the I κ B E3 ligase was then established by Pavletich and his colleagues, who solved the crystal structure of the substrate interacting pocket of β -TrCP in complex with the β -catenin phosphopeptide (sharing with I κ B α the destruction motif, i.e., degron) (Wu et al. 2003). β -TrCP was subsequently shown to control the proteasome-mediated degradation of other I κ Bs (I κ B β and I κ B ϵ) (Shirane et al. 1999; Wu and Ghosh 1999), and of NF- κ B1/p105 (Orian et al. 2000; Heissmeyer et al. 2001; Lang et al. 2003), as well as the signal-induced processing of NF- κ B2/p100 to p52 by the proteasome (Fong and Sun 2002).

THE I κ B E3 UBIQUITIN LIGASE

The half-life of I κ B α in resting cells is 138 min, two orders of magnitude longer than the half-

life of I κ B α in stimulated cells—1.5 min (Henkel et al. 1993). The signal that transforms the stable I κ B α in resting cells to the fast degrading form in stimulated cells is the phosphorylation of residues 32 and 36 (Chen et al. 1995; Yaron et al. 1997). Rapid degradation of the phosphorylated I κ B α demands an E3 with features allowing the speed, specificity, and inducibility that characterize I κ B α degradation. An SCF-type E3 possesses the required characteristics: (1) It recognizes mainly phosphorylated substrates, allowing recognition only after signal induced phosphorylation of the substrate, and (2) it has a variable receptor subunit, the F-box protein, which recognizes a wide spectrum of different substrates, permitting the necessary specificity (Deshaies 1999). The SCF complex forms a bridge between the E2 ubiquitin-conjugating enzyme and the different substrates. This interaction increases the effective concentration of the target lysine residues near the active site of the E2, thus catalyzing the ubiquitination of the attached substrate (Wu et al. 2003). The SCF complex is composed of the adapter protein Skp1 (Feldman et al. 1997), the scaffold protein cullin-1 (Feldman et al. 1997), the RING domain protein Rbx1 (Roc or Hrt1) (Skowyra et al. 1999) (an E2 adapter), and a variable F-box protein (FBP), which allows the broad specificity (Skowyra et al. 1997). All the FBPs share a conserved 40-amino-acid domain called the F-box domain, which connects to the rest of the SCF complex through Skp1 (Bai et al. 1996). The substrate-binding domain of the FBP is almost invariably positioned directly carboxy-terminal to the F-box domain in the sequence, and no FBP has more than one F-box domain (Cardozo and Pagano 2004). The FBPs are classified according to their substrate-binding motif. The FBW family that includes β -TrCP, which recognize I κ B, has a WD-40 repeats domain, structured as a β -propeller (Deshaies 1999). The WD-40 domain recognizes motifs containing phosphorylated Serines or Threonines, for example, the DpSGXXpS degron recognized by β -TrCP (Yaron et al. 1997; Smith et al. 1999). The FBL family has a Leucine-rich repeat in an

arc-shaped α - β -repeat structure. A nonobligatory preference of substrate recognition by FBL is the prephosphorylation of the substrate or, at times, its attachment to other proteins in a complex (Kobe and Kajava 2001). Other FBP are termed FBX and contain a variety of protein–protein interaction domains. It is very common for proteins in this family to recognize their substrates only following different post-translational modifications, such as glycosylation, hydroxylation, and others (Jaakkola et al. 2001; Yoshida et al. 2002; Yoshida et al. 2003). The three FBP families share the requirement for a substrate change before recognition, by either post-translational modification or binding of other proteins. This mode of regulation enables individual recognition of many substrates by the same FBP over different cellular conditions.

One of the best characterized FBPs is the FBW β -TrCP. This protein is located mainly in the nucleus (Davis et al. 2002), yet may show some activity also in the cytoplasm (Jiang and Struhl 1998). It binds an impressive list of phosphorylated substrates, including regulators of inflammation and cell fate (the I κ Bs), development and tissue organization (β -catenin, Snail), many cell cycle regulators (Emi1, Wee1), and DNA damage responders (Claspin, Cdc25A) (Jiang and Struhl 1998; Busino et al. 2003; Guardavaccaro et al. 2003; Watanabe et al. 2004; Zhou et al. 2004; Peschiaroli et al. 2006). In spite of β -TrCP's pivotal contribution to many oncogenic cellular pathways, there are not many known cases of mutations in the β -TrCP gene in human malignancies. This might reflect the essential physiological role of this E3 or redundancy of the two β -TrCP paralogues encoded by two different genes. The two paralogues are identical in their biochemical qualities because the “pocket” responsible for substrate recognition is identical (Fuchs et al. 1999). The redundancy may explain to the mild testicular phenotype observed in the in vivo ablation of β -TrCP1 (Guardavaccaro et al. 2003). The two best characterized substrates of β -TrCP, β -catenin and I κ B, do not accumulate in β -TrCP-deficient mouse embryonic fibroblasts (MEF ^{β -TrCP1^{-/-}}). The

phenotype detected in the knockout mice might stem from differential regulation of the two paralogues: For instance, β -TrCP2 may not be expressed in all cells of the testes at which the knockout phenotype has been noticed. The best way to validate the differential contribution of the two paralogs in vivo is probably a combination of inducible deletion of β -TrCP2 and β -TrCP1, which is currently underway in some laboratories.

REGULATION OF β -TrCP

β -TrCP is responsible for the degradation of many proteins in response to various stimuli, among which are cell cycle regulators, some with opposing functions. Each substrate demands the attention of β -TrCP in a different temporal, spatial, and physiological context. Some of the substrates, such as the I κ Bs, must be degraded quickly following stimuli, whereas others, like β -catenin, are continuously ubiquitinated by β -TrCP and must be stabilized on a stimulus. Therefore, β -TrCP must be available at all times and the main mode of its functional regulation has to be the recognition and binding to the different substrates in the right context. This feature is provided by the signal dependent phosphorylation of all β -TrCP substrates (Deshaies 1999; Karin and Ben-Neriah 2000; Nakayama and Nakayama 2005). Any regulation of β -TrCP itself must therefore be bounded to these limitations. There is, however, only limited data concerning the transcriptional regulation of β -TrCP. It was reported that β -TrCP1 is regulated by the Wnt pathway at the mRNA level, both by modulation of the rate of transcription and the stabilization of mRNA (Spiegelman et al. 2000; Ballarino et al. 2004). β -TrCP1 mRNA stability is also increased following Jun amino-terminal kinase (Jnk) activation in a transcription-independent manner (Spiegelman et al. 2002). The transcriptional regulation of β -TrCP2, however, is probably different, with Wnt stimulus leading to its transcriptional inhibition and mitogen-activated signaling resulting in a β -TrCP2 transcriptional increase (Spiegelman et al. 2002). There still

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remains the question of the physiological relevance of these controls modes, as unless the isoforms have different substrate specificity, opposing transcriptional regulation of the two may offset each other.

Taking into account the entire SCF complex as one functional E3 unit, it is difficult to predict the impact of the change in the level of one subunit on E3 function. A comparison between two transgenic mice, one overexpressing the WT β -TrCP1 and the other expressing a dominant negative mutant of β -TrCP1, exemplifies the problematics of up-regulation of the β -TrCP subunit by itself. The dominant negative mutant that lacks the F-box is unable to assemble an SCF complex, but rather sequesters β -TrCP substrates and hinders their recognition by WT β -TrCP. Yet, surprisingly, overexpression of WT β -TrCP caused the same degree of β -catenin accumulation and tumorigenesis rate (Belaidouni et al. 2005). Therefore, when WT β -TrCP is expressed in excess, the stoichiometry of the other components in the SCF complex is inadequate, resulting in the apparent dominant-negative function of WT β -TrCP transgene. The need for balanced expression of the SCF subunits likely rebuts the notion of modulating β -TrCP expression levels as a major mode of regulation of the E3 activity.

Acting as a part of the SCF complex, β -TrCP activity is also subject to regulation directed at other components of the complex. The Nedd8 ubiquitin-like molecule was found to regulate the assembly of the SCF complex (Wei and Deng 2003; Wolf et al. 2003). Nedd8 modification of Cul1 stabilizes the SCF complex by preventing the binding of Cand1. Cand1 binds to cullin-Rbx1 complex and occludes Skp1, thereby preventing SCF assembly (Liu et al. 2002). Cleavage of Nedd8 by the COP9/signalosome (CSN) will allow the binding of Cand1 and decrease the SCF activity (Lyapina et al. 2001). Thus, complex assembly provides another level of E3 regulation, preventing spurious activity of the E3 complex.

Another case of SCF regulation is evident in the interaction of β -TrCP with the abundant nuclear protein hnRNP-U, reminiscent of a

pseudo-substrate control, as hnRNP-U binds to the E3 but is not ubiquitinated (Davis et al. 2002). This may affect β -TrCP by maintaining it in the nucleus and raising its substrate-binding threshold. Raising the recognition threshold is an effective tool to avoid degradation of low affinity substrates, for example, none-, or partially phosphorylated substrates. Examples of a low affinity substrate are I κ B α molecules that are phosphorylated only at one of the two serines of the β -TrCP degron (Ser36 by IKK ϵ , and Ser32 by Rsk1) (Ghoda et al. 1997; Schouten et al. 1997; Peters et al. 2000): These will not compete effectively with saturating levels of hnRNP-U and will therefore escape β -TrCP ubiquitination.

These examples probably represent only the tip of the iceberg, as the intricate SCF structure provides ample room for activity regulation through multiple intrinsic (e.g., subunit modification) and extrinsic (accessory molecules) factors, mostly yet to be discovered.

I κ B RECOGNITION BY β -TrCP

Rarely there is as detailed a mechanistic understanding of the E3-substrate interaction as with β -TrCP. This understanding emerged from peptide inhibition studies, defining the degron sequence and the necessity for dual phosphorylation for ubiquitination of phosphorylated I κ B α (Yaron 1997). Yet a clearer interaction picture awaited the crystallography analysis of Pavletich and colleagues who pinpointed the critical features of substrate binding by β -TrCP (Wu 2003). This has been achieved by the cocrystallography of β -TrCP with a phosphorylated β -catenin peptide, sharing with I κ B the core degron motif DpSG Φ XpS (G Φ X referred to as a “spacer,” Φ stands for a hydrophobic, and X is any residue). The following binding details are observed in the structure: Phosphopeptide binding is mediated by one face of the β -propeller, a structure created by the seven WD repeat domain of β -TrCP; other domains of the F-box protein in the WD vicinity (e.g., the F-box, the F-box-WD linker, and the dimerization domain) or other SCF components do not participate in substrate

binding; a central groove running through the middle of the WD propeller structure accommodates the degron moiety of the substrate. Wu et al. noticed that all seven WD repeats of β -TrCP contribute contacts to the bound peptide. Nearly all of the β -TrCP contacts are made by the six-residue degron; the side chains of the Asp, the hydrophobic residue (Φ), the backbone of the Gly, and the spacer residues (X) insert the farthest into the groove, making intermolecular contacts in a mostly buried environment. The phosphate groups of the two serines bind sites at the rim of the groove and along with the Asp make the largest number of contacts with β -TrCP residues surrounding the groove through hydrogen bonds and electrostatic interactions. Whereas Pavletich's data explains well the interaction of β -TrCP with I κ Bs and other canonical degron motif substrates, a remaining question is how the E3 can accommodate peptides containing atypical degrons, such as p105, which contains a longer spacer. To that end, we used the Rosetta software (Das and Baker 2008) to model a loop of varying length and sequence onto the scaffold of the two phosphorylated serine anchors of the β -catenin peptide bound to β -TrCP (Wu et al. 2003). A comparison of the structural models of phospho-peptides derived from β -catenin (DpSGIHpS), I κ B α (DpSGLDpS), p105 (DpSGVETpS), and CDC25B (DpSGFCLDpS) suggests that these peptides of different spacer length can indeed all be accommodated into the groove (Fig. 1): Although the two phosphorylated serines are oriented similarly to those of β -catenin, the spacer between them—although of different length and sequence—displays similar characteristics. In all of the motifs, a glycine is followed by a hydrophobic residue (Ile, Leu, Val, and Phe) which is packed against a hydrophobic patch in the groove. The longer the binding motifs, the deeper into the groove will this hydrophobic residue be inserted. This advances our understanding of the principles that underlie substrate specificity, and in addition is invaluable for attempts to design β -TrCP blocking agents as NF- κ B inhibitors (see the following discussion).

DEGRADATION KINETICS OF THE DIFFERENT I κ Bs

The three I κ Bs (I κ B α , I κ B β , and I κ B ϵ) are degraded with different kinetics, I κ B α the fastest (approximately 15 min in Jurkat and THP-1 cells) and I κ B ϵ the slowest (120 min in Jurkat cells) (Whiteside et al. 1997). It is hard to define the factor that influences mostly these kinetics, and there might be a variation of the limiting factor in different physiological contexts. The first candidate is the stimulus itself, but even when examining one of the most effective stimuli, TNF α , there is hardly any correlation between the applied dose and the kinetics of IKK phosphorylation activity or I κ B degradation (Cheong et al. 2006). IKK itself can be the factor that distinguishes between the three I κ Bs. Mathematical models and experimental data indicated that NF- κ B dynamics are sensitive to the timing and duration of the IKK activity (Cheong et al. 2008). Indeed, the affinity between IKK and the different I κ Bs correlates with their degradation rate, implicating IKK as the main factor that shapes the differential kinetics (Heilker et al. 1999).

Another factor that might influence the degradation kinetics is the ubiquitination rate. Because the three I κ Bs share the same DSG degron, it is not plausible that the recognition by β -TrCP is discriminative, but perhaps other domains in the I κ B structure can influence the processivity of ubiquitination as with the different substrates of another E3 (Rape et al. 2006). The processivity of ubiquitination is influenced by the affinity to the E3 ligase and by the associated activity of deubiquitinating enzymes (DUBs) (Rape et al. 2006). Deubiquitination is often a major means of terminating ubiquitination-dependent signaling response, and could also function in reducing the basal, signal-independent ubiquitination of several proteins in NF- κ B signaling, including I κ Bs. CYLD and A20 are the two key DUBs in this regulation, but neither of them targets I κ Bs (Sun and Ley 2008). So far, no DUB has been found to negatively regulate the degradation of I κ B, but considering equivalent signaling systems (e.g., the DNA damage

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Figure 1. Accommodation of peptide degrons of different lengths into the β -TrCP binding groove. The β -TrCP WD40 domain is shown in white surface representation from a top view (1P22:A); the two phosphorylated Serine residues that are conserved among all the peptides are shown in stick representation; and different peptide backbones are colored according to the peptide: The β -catenin peptide is shown in red (1P22:C); a model of the $\text{I}\kappa\text{B}\alpha$ peptide (DSGLDS) is shown in blue; p105 peptide (DSGVETS) in yellow; and CDC25B peptide (DSGFCLDS) in green. Note that with increasing length of the spacer between the two phosphorylated serine residues, the hydrophobic part of the peptide binds deeper into the pocket.

response) (Zhang et al. 2006; Bassermann et al. 2008), it may exist, providing an important regulatory aspect to NF- κ B signaling.

The unique degradation characteristics of the $\text{I}\kappa\text{B}$ s fit their nonredundant roles in the regulation of the NF- κ B response. It is best exemplified in the computational model generated by Hoffmann et al. (2002). Using three mouse models in which each $\text{I}\kappa\text{B}$ was deleted separately, it was shown that $\text{I}\kappa\text{B}\alpha$ is inimitable in inducing a rapid, strong negative feedback regulation, resulting in an oscillatory NF- κ B activation profile. $\text{I}\kappa\text{B}\beta$, and particularly $\text{I}\kappa\text{B}\epsilon$, respond more slowly to IKK activation and act to dampen the long-term oscillations of the NF- κ B response. The interplay between these isoforms dictates the onset and termination of NF- κ B activation, allowing a relatively stable NF- κ B response during long-term stimulation. Consistent with the major role of $\text{I}\kappa\text{B}\alpha$, deletion

of $\text{I}\kappa\text{B}\beta$ and $\text{I}\kappa\text{B}\epsilon$ in animals is mostly harmless, indicating that other $\text{I}\kappa\text{B}$ s can compensate for their loss (Memet et al. 1999; Hoffmann et al. 2002). In contrast, deletion of $\text{I}\kappa\text{B}\alpha$ resulted in constitutive NF- κ B activation and embryonic lethality (Beg et al. 1995), yet, once $\text{I}\kappa\text{B}\beta$ is expressed under the promoter of $\text{I}\kappa\text{B}\alpha$, it provided full compensation for the absence of $\text{I}\kappa\text{B}\alpha$ (Cheng et al. 1998). This finding highlighted the importance of unique transcriptional regulation of $\text{I}\kappa\text{B}\alpha$ rather than its unique activity or its own degradation rate, in controlling NF- κ B activity.

In addition to their distinctive degradation kinetics, the three $\text{I}\kappa\text{B}$ s show different quantitative contribution to the NF- κ B response. Unfortunately, a quantitative model based on the experimental data cited above failed to distinguish between the three $\text{I}\kappa\text{B}$ s (Cho et al. 2003). Thus, this aspect still awaits the

formulation of a proper underlying model. In particular, the relative stoichiometry of association of each I κ B with NF- κ B is not known. Nevertheless, there is a negative correlation between the relative concentration of nuclear NF- κ B and the overall cellular I κ B α levels, indicating a major role of I κ B α in controlling the NF- κ B localization (Pogson et al. 2008). It will be interesting to study NF- κ B localization with respect to the relative concentrations of the other I κ Bs, and thus incorporate further kinetic and quantitative aspects into a unified model of NF- κ B activation.

UBIQUITINATION OF p100 AND p105 CONTROLS THEIR I κ B FUNCTION

p100 and p105 also function as I κ Bs (Rice et al. 1992; Mercurio et al. 1993) but do not share the main features of a canonical degradation pathway. Their primary function is to serve as precursors for the mature NF- κ B proteins p52 and p50, a function regulated by proteasomal processing (Fan and Maniatis 1991). Yet, the ankyrin repeat structure of p100 and p105 binds NF- κ B/Rel proteins, similarly to the homologous region of the “professional I κ Bs” (α , β , and ϵ), and can therefore fulfill an I κ B function, assuming that the fraction of NF- κ B bound to these precursor proteins is sometimes free to dissociate, enter the nucleus, and induce transcriptional activity. In this respect, precursor processing regulates the NF- κ B pathway in two planes: producing new and releasing old NF- κ B molecules for the benefit of the transcriptional response. p100 is phosphorylated by IKK α that is recruited and activated by NF- κ B-inducing kinase (NIK) as part of the noncanonical NF- κ B signaling pathway (Xiao et al. 2001; Xiao et al. 2004). This phosphorylation promotes the binding of β -TrCP, polyubiquitination, and proteasomal processing, which generates active, I κ B-free p52 (Fong and Sun 2002). In contrast, p105 is constitutively processed both in resting and stimulated cells (Weil et al. 1999; Orian et al. 2000), a course of action mediated by an independent, yet to be identified E3 that induces mono-ubiquitination rather than the polyubiquitination mediated by

β -TrCP (Kravtsova-Ivantsiv et al. 2009). Still, the inhibitory function of p105 is compliant with canonical IKK β phosphorylation, and p105 proteasomal processing, which is facilitated by signaling (Mercurio et al. 1993) reduces the inhibition (Heissmeyer et al. 1999). In addition, a fraction of p105 is targeted for complete degradation following certain cell stimuli, such as TNF α (Lang et al. 2003).

Overexpressed p105 was found to sequester c-rel and p65 in the cytoplasm of COS-7 and CV-1 cells (Rice et al. 1992). Endogenous p105 and p100 were shown to associate with c-rel and p65 in Jurkat cells (Mercurio et al. 1993) and in WT MEFs in vivo (Basak et al. 2007). Unfortunately, in these studies, the question of whether the binding of p100 or p105 is quantitatively significant has not been addressed and the stoichiometry of the NF- κ B fraction that is bound to these proteins is unknown. The RelA fraction that is bound to p100 or p105 could vary from cell to cell and be subject to cell stimulation or other cellular conditions. Thus, its quantification might provide a better understanding of the I κ B role played by the NF- κ B precursors.

A study focusing on the noncanonical NF- κ B signaling downstream to the LT β R provided evidence that p100 functions as an inhibitor in I κ B α ^{-/-}, I κ B β ^{-/-}, and I κ B ϵ ^{-/-} MEFs (Basak et al. 2007). This work also showed that the fraction of p100-bound, dissociable p65 was increased three- to fourfold following TNF α stimulation, proposing a specific time window for I κ B function of p100 after canonical signaling in WT cells. This suggestion was further supported by transcriptional synergism between LT β R and TNF α . However, some relevant questions were left unanswered: whether the overall stoichiometry of p100-bound NF- κ B is significantly changed following canonical stimulation, and whether the transcriptional synergism between LT β R and TNF α stimuli brought as evidence for an I κ B function of p100 could in fact be caused by IKK activation with LT β R (Chang et al. 2002).

An in vivo research of the inhibitory role of p100 and p105 is hard to conduct because most genetic manipulations will also influence p50

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and p52 and thus, it will be hard to distinguish the I κ B-relevant effects. One way around this point is to stabilize a precursor by way of site directed mutagenesis, which will spare processing. Indeed this was recently achieved in the case of p105 in T cells, showing that substitution of the modifiable serine residues controlling p105 stability resulted in a moderate “I κ B super-repressor”-like molecule, affecting T-cell development (Sriskantharajah et al. 2009). However, again the stoichiometry of p105-bound NF- κ B is nonphysiological, thus not necessarily attesting to a physiological I κ B function of p105 in T cells. As a heterozygous mutation that substitutes human Ser32 of I κ B α is sufficient to cause T-cell immunodeficiency and ectodermal dysplasia (Courtois et al. 2003; Janssen et al. 2004), it is possible that equivalent serine substituting mutations in the *NFKB1* and *NFKB2* genes will cause similar human pathologies, thereby supporting the I κ B function of NF- κ B precursor protein.

To conclude, better understanding of the true I κ B role of p100 or p105 requires quantitative and kinetic studies, with comparison to the “professional” I κ Bs, preferentially under physiologic conditions (i.e., in WT cells in response to various stimuli) (see Fig. 2). This will probably be best achieved with the help of mathematical modeling. Only then may we understand the role of each I κ B in physiological NF- κ B response.

SUBCELLULAR LOCATION OF I κ B UBIQUITINATION AND DEGRADATION

A large missing piece in the NF- κ B activation scheme is the site of I κ B α ubiquitination and degradation. This issue is underscored by the discordant subcellular localization of the I κ Bs, both the “professionals” and the NF- κ B precursors, which are mostly cytoplasmic, and the E3 β -TrCP, which is mostly nuclear. A major fraction of β -TrCP is sequestered in the nucleus by the pseudo-substrate hnRNP-U, and co-immunoprecipitation studies show that this hnRNP-U- β -TrCP complex is available for ubiquitinating I κ B α on exchange of the low affinity hnRNP-U pseudosubstrate by the high affinity pI κ B α (Davis et al. 2002). Apart from

the β -TrCP, other components of the SCE, as well as the proteasome, are localized and active in the nucleus (Nakayama and Nakayama 2005; von Mikecz 2006). Nuclear proteasome substrates include the p53 inhibitor Mdm2, the CDK inhibitor Far1, and others (von Mikecz 2006). How can we therefore envision the interaction between a nuclear E3 and cytoplasmic substrates? One way is to assume that the location of the components is not stable, and at least one component, the substrate or the E3, may switch its subcellular location randomly or on demand. It was therefore interesting to note that I κ B α -NF- κ B complexes shuttle continuously between the cytoplasm and the nucleus of unstimulated cells. The shuttling is mediated by a nuclear export signal (NES), located near the amino terminus of I κ B α , and a likely unmasked nuclear localization signal (NLS) of the p50 proteins (Malek et al. 2001). I κ B β , which lacks the NES, is probably more efficient in masking the NLS because I κ B β -NF- κ B complexes are found exclusively in the cytoplasm (Huang and Miyamoto 2001). I κ B α shuttling is apparently unperturbed as long as the NF- κ B/I κ B α complex is stable. Once I κ B α is phosphorylated via activated IKK, it is recognized by β -TrCP and degraded, following which NF- κ B is retained in the nucleus. Whereas phosphorylation of I κ B α is thought to occur in the cytoplasm (Karin and Ben-Neriah 2000), the subcellular site of its ubiquitination and degradation is unknown.

It is important to note that although the I κ B α /NF- κ B shuttling is a reasonable solution for introducing a nuclear E3 to a cytoplasmic substrate, most of the relevant experiments have relied on LMB treatment as an inhibitor of I κ B α nuclear export. Other influences of this drug that may affect NF- κ B signaling have never been taken into account. Therefore, any activation model entailing steady state shuttling must be taken with a grain of salt.

THE ROLE OF THE I κ Bs IN TERMINATION OF NF- κ B SIGNALING

Temporal accuracy is required for induction and for termination of NF- κ B activity. One of

Figure 2. Ubiquitin-mediated proteolysis at the central stage of the NF- κ B. Many regulatory steps in the NF- κ B pathway involve ubiquitin-regulated proteolysis. In resting cells (*right panel*), basal level processing of the NF- κ B1 precursor p105 to p50, mediated by a yet unidentified E3(s) (E3x), is the major proteolytic event. The processing product p50 associates with p65 to generate the NF- κ B heterodimer, maintained inactive in the cytosol through association with an inhibitor: one of the “professional I κ Bs” or an NF- κ B precursor (both p105 and p100). On cell stimulation (*left panel*), TNF α and LT α / β signal-mediated degradation of the I κ Bs and processing NF- κ B2 (p100) to the mature p52 subunit, respectively, are the key proteolytic steps. All these ubiquitin-proteolysis events are controlled by a single E3 ubiquitin ligase, β -TrCP. A prerequisite for β -TrCP-induced ubiquitination is phosphorylation of its target proteins, mediated by IKK (not shown). Following I κ B degradation, NF- κ B accumulates in the nucleus and is free to induce transcriptional activation. Another major feature of the NF- κ B pathways is the negative feedback control (red lines) by which first the newly transcribed I κ B α and then the NF- κ B precursors function to shorten signal duration by inhibiting NF- κ B-controlled transcription. Pairing I κ B α transcription and resynthesis to its rapid degradation shapes an oscillatory NF- κ B response, which might have a particular regulatory significance, yet to be discovered. I κ B ϵ transcription by NF- κ B function to reduce the oscillatory magnitude of the response and stabilize it during longer stimulations.

the major factors that controls the lifetime of the NF- κ B response is a negative feedback loop based on NF- κ B-dependent I κ B synthesis (Le Bail et al. 1993). Following a near-complete signal-induced degradation of I κ B, the newly synthesized I κ B α enters the nucleus and pulls NF- κ B away from the chromatin and back to the cytoplasm (Arenzana-Seisdedos et al.

1995). This process should allow a sufficient period of NF- κ B activity to induce relevant target genes and prevent the extension of the transcriptional activity beyond the necessary time, to avoid deleterious overactivation effects (Hoffmann et al. 2002). It has been argued that NF- κ B modification is one of the key factors that prevents premature termination

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of the transcriptional activity. For instance, RelA acetylation could prohibit its interaction with I κ B, whereas its deacetylation by HDAC3 increases its affinity to I κ B, thus allowing the eviction of NF- κ B from the chromatin by the newly synthesized I κ B α . Indeed, a timely acetylation–deacetylation cycle was shown to contribute to signal maintenance for 45 min (Chen et al. 2001). Following TNF α stimulus, NF- κ B is activated for approximately 45 min (Hoffmann et al. 2002), whereas I κ B α is transcribed as early as 15 min and peaks within 1 h (Scott et al. 1993). However, IKK activity peaks after 15 min and is maintained for more than an hour (Cheong et al. 2006), a period during which all the newly synthesized I κ B α could be immediately phosphorylated by IKK and subsequently degraded. How then can I κ B exert its termination role in the presence of an active IKK?

One possibility is that both IKK α and IKK β phosphorylate NF- κ B-bound I κ B more efficiently than free I κ B (Zandi et al. 1998), explaining how the free I κ B can accumulate in IKK-active cells. Nevertheless, a major fraction of the newly synthesized I κ B appears phosphorylated (Werner et al. 2005), suggesting that phosphorylation would not be the rate-limiting step in the destruction of this newly synthesized molecule. Another possibility is reduced ubiquitination of the unbound I κ B by β -TrCP. Unlike many SCF substrates, most lysine residues of I κ B are not targeted for ubiquitination in vivo, nor in the bound state in vitro, and the only degradation-relevant residues are Lys 21 and 22 of I κ B α (Scherer et al. 1995). Lys 21 is also the target residue for sumoylation, a modification that might antagonize proteolysis-marking ubiquitination of I κ B and could spare the newly synthesized I κ B if happening soon after synthesis. However, sumoylation was shown to happen only in the nucleus (Rodriguez et al. 2001), which similarly to, or in parallel with RelA acetylation, might imply a role in terminating the NF- κ B response, merely by stabilizing nuclear I κ B α . Overexpressed Sumo-1 blocked TNF α and IL-1 dependent ubiquitination and degradation of I κ B α in COS7 cells (Franzoso et al. 1992), yet S32/36

phosphorylated I κ B α was resistant to sumoylation in vitro, possibly indicating the relevance of this modification only for the newly synthesized, nonphosphorylated I κ B.

One should bear in mind that an unbound I κ B could also be targeted by E3s other than β -TrCP, which do not require IKK phosphorylation (Yaron et al. 1997; O'Dea et al. 2008) and might be degraded as efficiently as phosphorylated I κ B (O'Dea et al. 2008). I κ B α can also be phosphorylated independently of a signal on its carboxyl terminus by the kinase CK2 (Barroga et al. 1995) or in response to a noncanonical stimulus, such as UV (Kato et al. 2003). Knockdown of the CK2 β subunit or mutation of the phosphorylated residues in the carboxy-terminal fragment of I κ B α abrogated the NF- κ B response following UV (Kato et al. 2003). In addition, in response to doxorubicin-induced DNA damage, I κ B α undergoes proteasomal degradation, both in WT and in IKK α ^{−/−} and IKK β ^{−/−} MEFs (Wu and Miyamoto 2007). If this is physiologically relevant, it basically means that we do not understand how the newly synthesized I κ B, whether phosphorylated or not, escapes ubiquitination and degradation, and there must be a mechanism to seclude it from the ubiquitination machinery or reverse its ubiquitination effectively (i.e., by deubiquitination).

THERAPEUTIC IMPLICATIONS OF I κ B DEGRADATION

Aberrant NF- κ B regulation has been implicated in autoimmune diseases and in certain types of cancer. Given their central role of regulating NF- κ B activation, the proteins involved in ubiquitination and degradation of the I κ Bs are attractive targets for drug development. The successful treatment of a major hematological malignancies using a proteasome inhibitor has validated the ubiquitin–proteasome system as therapeutic target (Rajkumar et al. 2005). Bortezomib (formerly known as PS-341), a boronic acid dipeptide that binds directly with and inhibits the proteasome enzymatic complex, has a significant therapeutic activity in the treatment of advanced multiple myeloma

(MM) (Hideshima et al. 2002) and was approved for MM treatment by the FDA in 2003. MM is often characterized by NF- κ B pathway mutations (Annunziata et al. 2007; Keats et al. 2007) and Bortezomib's effects on cells are partly mediated through NF- κ B inhibition (Russo et al. 2001; Hideshima et al. 2002), resulting in apoptosis, decreased angiogenic cytokine expression, and inhibition of tumor cell adhesion to stroma. However, continuous proteasomal inhibition is toxic and therefore, bortezomib cannot be administered more than twice weekly, with proteasome inhibition lasting approximately 24 h after each injection (Adams 2002). An alternative approach to inhibiting NF- κ B is to block I κ B ubiquitination. This would be expected to have less undesired side effects than global proteasome inhibition and may be particularly attractive for cancer therapy (Fuchs et al. 2004). Here, too, one could believe in blocking a class of relevant E3s, or targeting specifically β -TrCP. Recent studies indicate the feasibility of blocking SCF-type E3s through interference with their activation requiring cullin modification by the ubiquitin-like molecule Nedd8 (Soucy et al. 2009). A more specific strategy for NF- κ B inhibition would be selective I κ B stabilization. The validity of this approach has been established in numerous experiments, both in vitro and in vivo, using a dominant I κ B "super-repressor," which cannot be inducibly phosphorylated and ubiquitinated but maintains its NF- κ B inhibitory capacity (Cusack et al. 2000; Lavon et al. 2000). For example, the expression of such an inhibitor in liver cells has been shown to halt tumor growth in a murine model of hepatitis-associated cancer in vivo (Pikarsky et al. 2004). A clinically relevant method for that purpose is to prevent I κ B ubiquitination or to impair β -TrCP recruitment to I κ B. Cell-penetrating I κ B phosphopeptides have been used for this purpose in cell lines (Yaron et al. 1997), but their efficacy has yet to be shown in vivo. It might also be possible to develop small-molecule inhibitors that structurally mimic the ligase recognition motif or specifically inactivate SCF $^{\beta\text{-TrCP}}$ (through allosteric inhibition or disassembly). One obvious

drawback with targeting β -TrCP is the potential to affect SCF $^{\beta\text{-TrCP}}$ targets other than I κ Bs (Spiegelman et al. 2002). A critical issue is likely to be whether SCF $^{\beta\text{-TrCP}}$ is the only E3 that contributes to the degradation of any given substrate. One obvious concern is stabilization of β -catenin, an important factor in colorectal tumorigenesis and other cancer types (van Es et al. 2003). One possibility, however, is that whereas I κ B degradation relies exclusively on β -TrCP (see Fig. 2), other substrates like β -catenin have alternative ubiquitination and degradation routes, operating on β -TrCP inhibition. Consistent with this hypothesis, a distinct E3 complex containing the Siah RING finger protein has been shown to recognize and promote the degradation of β -catenin independently of SCF $^{\beta\text{-TrCP}}$ (Liu et al. 2001; Matsuzawa and Reed 2001). Future analysis of β -TrCP-deficient mice will air this issue, proving whether the NF- κ B pathway is affected in these mice far more than other signaling pathways. Furthermore, even if the selective inhibition of I κ B ubiquitination is a technically feasible goal, systemic inhibition of NF- κ B is not without risk because of its crucial role in regulating immune responses (Caamano and Hunter 2002). This is a particularly important consideration when NF- κ B blockade is considered in combination with standard chemotherapy, which itself compromises the immune system and exacerbates tissue damage (Lavon et al. 2000). Furthermore, NF- κ B inhibition may in fact be a "double-edged sword," as in the presence of a carcinogen, and perhaps even under chemotherapeutic poisoning, NF- κ B inhibition may facilitate, rather than prevent, tumor development (Kamata et al. 2005).

CONCLUDING REMARKS

Inducible degradation of I κ B has become a paradigm of targeting a regulatory protein by the ubiquitin proteasome system as a major step in the activation of a signaling pathway. A central feature of this process is the coupling of two protein modification events, substrate bi-phosphorylation by a dedicated kinase,

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IKK, and ubiquitination, based on recognition of the phosphorylated degron by the E3. However, NF- κ B is regulated by five different inhibitory proteins (I κ B α , β , and ϵ and the two I κ B-like NF- κ B precursors), each holding some portion of NF- κ B in an inactive state. Therefore, maximal activation of the pathway requires simultaneous elimination of all five I κ Bs. This may be achieved on costimulation of both the canonical and alternative branches of the pathway, inducing degradation and processing of different I κ Bs, respectively. No wonder that the coordination of the degradation response relies on a similar, IKK-induced phospho-degron that is shared among all I κ Bs and on a single E3, β -TrCP, which evolved to fit the nuances of the degron in the different I κ Bs.

Despite of the major advances achieved in understanding ubiquitin-signaling processes of NF- κ B activation, several key questions remain unanswered, for example: What is the relative role of each I κ B in controlling NF- κ B activation in response to different stimuli? How is the NF- κ B-transcribed, newly synthesized I κ B protected from degradation as long as IKK is still activated? Which parameters other than phosphorylation affect the rate of degradation and processing of the different I κ B? Where in the cell are I κ B ubiquitination and proteolysis taking place? How important is the regulation of the ubiquitin–proteasome machinery itself in NF- κ B signaling? Is I κ B ubiquitination also regulated by specific deubiquitination enzymes? Are there any specific proteasome adaptor molecules that ensure degradation of I κ Bs, while protecting associated NF- κ B from proteolysis by the proteasome? How frequently are mutations in the I κ Bs or their destruction machinery encountered in human disease?

Considering these and other outstanding issues, the regulation and function of ubiquitination are likely to remain a major focus of research in the NF- κ B field.

ACKNOWLEDGMENTS

Relevant research in the authors' laboratories was supported by the Israel Science Foundation,

the RUBICON Network of Excellence of the European Commission (FP6), The German Israeli Foundation, and the Adelson Medical Research Foundation.

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