Ubiquitin signals in the NF-κB pathway

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Abstract

The NF-κB (nuclear factor κB) transcription factors control cell survival, proliferation and innate and adaptive immune response. Post-translational modifications of key components of the NF-κB pathway provide the molecular basis for signal transmission from the cell membrane to the nucleus. Here, we describe the involvement of different types of ubiquitin modification in the regulation of the NF-κB signalling pathway.

Introduction

The NF-κB (nuclear factor κB) transcription factors are key regulators of immune response, cell survival and proliferation, but they also play important roles in development of human diseases such as cancer development and progression [1]. NF-κB transcription factors function as a dimer of five different NF-κB proteins: RelA (p65), RelB, c-Rel, p50 and p52. All of these proteins possess an RHD (Rel-homology domain), which is composed of dimerization, nuclear-localization and DNA-binding domains. Under the non-stimulated condition, NF-κB proteins are dimerized and rendered inactive either by their inhibitor IκB (inhibitory κB), which holds them in the cytoplasm, or they are kept inactive as unprocessed, precursor proteins. Cell stimulation activates IKK (IκB kinase) that phosphorylates IκB, which recruits the E3 ubiquitin ligase SCF (Skp1/cullin/F-box)–βTrCP to IκB, resulting in its ubiquitination and proteasomal degradation. In this way, NF-κB is released and can enter the nucleus where it regulates the transcription of different classes of genes, such as cytokines, pro- and anti-apoptotic genes, growth factors, adhesion molecules, matrix proteases, cell cycle genes and angiogenic factors [2,3].

Depending on the type of kinase being activated by an appropriate signal, the NF-κB activation pathway is named the classical (canonical) pathway, involving mostly IKKβ, or the alternative (non-canonical) pathway, which is strictly dependent on IKKα. The classical pathway is the predominant way of activating NF-κB transcription factors and is usually triggered in response to microbial infection or pro-inflammatory cytokine stimulation, whereas the alternative pathway is induced by some members of the TNFR [TNF (tumour necrosis factor) receptor] family. The NF-κB signalling pathway can be activated by many stimuli including microbes, inflammatory cytokines, stress, necrotic cell products and antigen stimulation [via TCR (T-cell receptor)]. Most of the biological processes affected by NF-κB signalling play central roles in carcinogenesis, making NF-κB signalling and its regulation a major target of cancer research [4].

Both the classical and the alternative NF-κB activation pathways critically depend on ubiquitin signalling [5,6]. Ubiquitin is a small (8 kDa) protein that can be covalently and reversibly attached to lysine residues of target proteins in a process called ubiquitination. Initially, ubiquitin is activated by a ubiquitin-activating enzyme E1 in an ATP-dependent process followed by its transfer to a ubiquitin-conjugating enzyme E2. The final step of the ubiquitination reaction is carried out by the ubiquitin-protein ligase E3, which attaches ubiquitin to the lysine residue in the target protein [7]. Ubiquitin modifications of proteins can occur in multiple forms [8]. In the simplest way, a single ubiquitin molecule is attached to a single lysine residue in a substrate, which is defined as mono-ubiquitination. When several single ubiquitin molecules are attached to several different lysine residues, the process is called multiple mono-ubiquitination or multi-ubiquitination. Moreover, ubiquitin can be added to lysine residues in the ubiquitin molecule itself, thus forming ubiquitin chains in a process known as polyubiquitination. Proteins that are polyubiquitinated with Lys^{48}-linked chains are targeted for proteasomal degradation. Interestingly, ubiquitination of many proteins does not mediate their degradation but rather has functional consequences for the modified protein. These include changes in their conformation, subcellular localization or catalytic activity. In addition, the attachment of ubiquitin can engage them in protein–protein interactions with effectors containing UBDs (ubiquitin-binding domains). This leads to the formation of ubiquitin signalling networks, which translate signals from the cellular environment into proper biological phenotypes [8]. In analogy to phosphorylation, ubiquitination is also a dynamic and reversible modification. The rapid removal of ubiquitin from substrates is catalysed by DUBs (de-ubiquitinating enzymes).

Key words: de-ubiquitinating enzyme, inhibitory κB kinase (IKK), nuclear factor κB (NF-κB), transcription factor, tumour necrosis factor receptor (TNFR), ubiquitination.

Abbreviations used: NF-κB, nuclear factor κB; ABIN, A20-binding inhibitor of NF-κB; CYLD, cylindromatosis; DUB, de-ubiquitinating enzyme; IκB, inhibitory κB; IKK, IκB kinase; IL-1, interleukin-1; IL-1R, IL-1 receptor; IRAK1, IL-1R-associated kinase 1; LZ, leucine zipper; MyD88, myeloid differentiation factor 88; NEMO, NF-κB essential modulator; NOD, nucleotide-binding oligomerization domain; RHD, Rel-homology domain; RIP, receptor-interacting protein; SCF, Skp1/cullin/F-box; TGF, transforming growth factor; TAK1, TGF-β-activated kinase 1; TAB1, TAK1-binding protein; TCR, T-cell receptor; TLR, Toll-like receptor; TNF, tumour necrosis factor; TNFR, TNF receptor; TRADD, TNFR-associated via death domain; TRAF, TNFR-associated factor; TRIKA, TRAF-6-regulated IKK activator; UBC, ubiquitin-conjugating enzyme; UBD, ubiquitin-binding domain; ZAP-70, ζ-chain (TCR)-associated protein kinase of 70 kDa.

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Positive regulators of ubiquitin signals

The NF-κB pathway is regulated by diverse ubiquitination modifications. Some of these reactions have activating outcomes, whereas the others down-regulate NF-κB signalling. Following receptor stimulation, TRAFs (TNFR-associated factors) are among the first molecules to be engaged in signal transmission (Figure 1). TRAF proteins are ubiquitin E3 ligases, which have a pivotal role in the activation of NF-κB signalling pathway via TLRs (Toll-like receptors), IL-1R [IL-1 (interleukin-1) receptor] and the TNFR superfamily [9]. For example, TRAF-2 is recruited to the activated TNFR via the action of the adaptor protein TRADD (TNFR-associated via death domain) and through the interaction with RIP (receptor-interacting protein). TNFα stimulation induces Lys^{63}-linked polyubiquitination of RIP, which then associates with TNFR as well as the regulatory protein IKKγ/NEMO (NF-κB essential modulator), which activates the IKK complex [10].

On the other hand, TRAF-6 is essential for the IKK activation following stimulation of the IL-1R and TLRs (Figure 1). Activation of IL-1R or TLR receptors recruits an adaptor protein, MyD88 (myeloid differentiation factor 88) to the receptor, which attracts the protein kinases IRAK1 (IL-1R-associated kinase 1) and IRAK4 to the complex. IRAK1 binds to MyD88 and to TRAF-6, which in turn activates the IKK complex [11]. However, TRAF-6 does not activate IKK directly but rather via its two activators TRIKA1 (TRAF-6-regulated IKK activator 1) and TRIKA2 [9]. TRIKA1 is a ubiquitin-conjugating E2 enzyme complex consisting of UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin E2 variant 1A) proteins. TRIKA1 functions together with TRAF-6 (an E3 enzyme) to synthesize a Lys^{63}-polyubiquitin chain on target proteins including TRAF-6 itself, the regulatory protein NEMO and possibly other proteins. TRIKA2 is a protein complex consisting of TAK1 [TGF-β (transforming growth factor-β)-activated kinase 1], TAB1 (TAK1-binding protein) and TAB2. Mutations within the NZF (Nlp14 zinc finger), the UBD of TAB2, abolishes its ability to activate TAK1 and IKKβ, suggesting that TAB2 binds to ubiquitinated TRAF-6 (already in complex with activated receptor) prior to activating TAK1 [9]. It seems that TAB1 protein is not very important for IKK activation since deletion and silencing studies of this protein did not impair IKK functions. On the other hand, TAB2 facilitates TRAF-6 ubiquitination by IL-1 stimulation, which promotes the assembly of TRAF-6 with the downstream kinase, IKK [12].

In addition, NF-κB transcription factors play an important function in immune responses towards specific antigens by TCR (Figure 1). Following the activation, TCR is recruited to rafts with Bcl-10 and MALT1 (mucosa-associated lymphoid tissue lymphoma translocation gene 1) proteins that induce Lys^{63}-linked polyubiquitination of NEMO (probably by TRAF-6), leading to the activation of IKKs [13,14]. Some bacterial receptors are located in the cytoplasm; NOD (nucleotide-binding oligomerization domain) 1 and NOD2 are two such receptors. The main outcome of their engagement with ligand is NF-κB activation, which almost exclusively occurs via RIP2. Following NOD2 activation, RIP2 mediates Lys^{63}-linked polyubiquitination of NEMO, which is followed by phosphorylation of IKKβ and activation of the NF-κB pathway [15].

NEMO is not only polyubiquitinated but also binds specifically to Lys^{63}-linked polyubiquitin chains via its UBD [10,16]. It was shown that NEMO binds to polyubiquitinated RIP2 following TNF stimulation. The UBD was determined to be the coiled-coil LZ (leucine zipper), which is critical for TNFα-induced NF-κB activation. A mutation at Lys^{329}, which is a critical amino acid for ubiquitin binding and is located in the LZ, is sufficient to block the TNFR1-mediated recruitment of IKK to TNFR1. In such ways, NEMO functions as an essential mediator of NF-κB signalling.

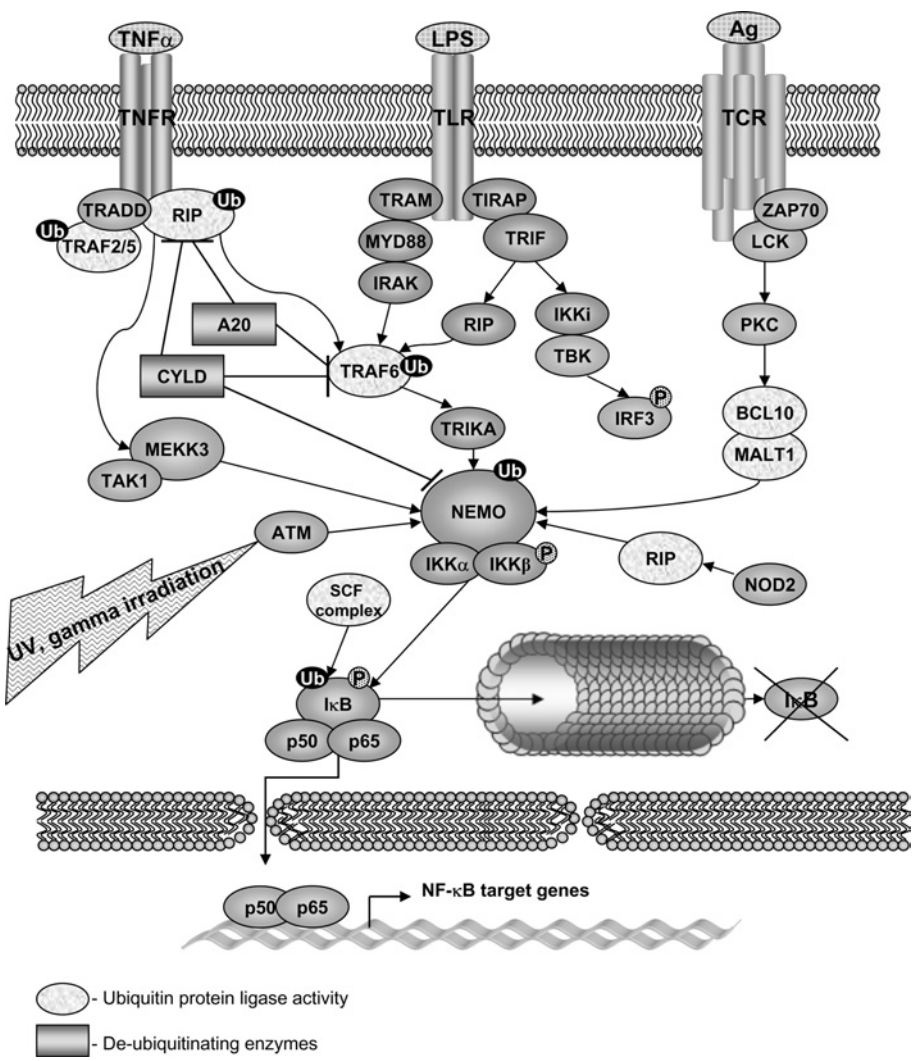
Ubiquitin-mediated activation of NF-κBs

NF-κBs are kept in the cytoplasm in the inactive form in two ways: bound to their inhibitor IκB or as inactive precursor proteins. Activation of NF-κB proteins is initiated by the phosphorylation of their inhibitors by IKK proteins. Following phosphorylation, IκB proteins are polyubiquitinated via Lys^{48} and are thus destined for proteasomal degradation. Ubiquitination of IκB is performed by an E2 ubiquitin-activating enzyme of the UBC4/5 family followed by E3 ligase action of SCF–βTrCP. There are two different E3 ligases involved in this process, βTrCP1 and βTrCP2, which have overlapping functions since genetic deletion or silencing of one of the genes only partially blocks IκBα degradation [17,18]. The ligases bind only to the phosphorylated form of the IκBα through their C-terminal part, which allows the conjugation of ubiquitin to two conserved N-terminal lysine residues. The ubiquitinated IκBα remains associated with NF-κB proteins and is selectively degraded in the proteasome, whereas (non-ubiquitinated) NF-κB is spared from degradation and released from the inhibitory partner.

Most of the NF-κB precursor proteins such as p105 are co-translationally processed in a way that depends on the proteasome only without preceding phosphorylation or ubiquitination. Also, cell stimulation with LPS (lipopolysaccharide) can activate the IKK complex that phosphorylates p105, inducing its processing. On the other hand, processing of the precursor protein p100 is always dependent on phosphorylation/ubiquitination. p100 is phosphorylated at a specific serine residue and polyubiquitinated by SCF–βTrCP, which induces its proteasomal processing to the mature p52 protein, enabling it to translocate into the nucleus [19]. Both of the proteins, p100 and p105, are only partially degraded at their C-terminal part that harbours IκB-like domains. It is uncertain how the proteasome decides whether to degrade a protein completely or only partially. In the case of p100 and p105, it seems that ubiquitin tagging of the precursor proteins attracts the proteasome to the internal, glycine-rich region where proteolysis starts and proceeds to RHD, which is difficult to unfold, which makes it difficult to degrade [20].

Figure 1 | Ubiquitin modification in the NF-κB pathway

The classical NF-κB pathway can be triggered by activating several families of cell-surface receptors (TNFR and TLR). Activation of TNFR results in recruitment of TRADD, TRAF (TRAF-2 or -5) and RIP to the receptor and subsequent Lys^{63}-polyubiquitination of RIP. Activated RIP stimulates TRAF-6, a ubiquitin E3 ligase and crucial in NF-κB activation. TRAF-6 is also activated via TLR triggering, through MyD88 and IRAK interaction. TRAF-6 together with TRIKA induces Lys^{63}-polyubiquitination of NEMO and TRAF-6. Activation of TCR recruits Bcl-10 and MALT1 (mucosa-associated lymphoid tissue lymphoma translocation gene 1) ubiquitin ligases, which, together with TRAF-6, ligate a Lys^{63}-polyubiquitin chain to NEMO. NEMO activation results in IKKβ phosphorylation, resulting in proteasomal IκB degradation after phosphorylation and Lys^{48}-polyubiquitination (by the SCF complex). Now, NF-κB dimers are free to enter the nucleus and activate gene transcription. De-ubiquitination enzymes, A20 and CYLD, de-ubiquitinate Lys^{63}-chains from target molecules. Also, A20 forms a Lys^{48}-ubiquitin chain on RIP and therefore can induce its proteasomal degradation. IRF-3, interferon regulatory factor-3; TBK, TANK (TRAF-associated NF-κB activator)-binding kinase; TRAM, TRIF [TIR (Toll/IL-1 receptor) domain-containing adaptor protein inducing interferon β]-related adaptor molecule; Ub, ubiquitin.



Negative signalling in the NF-κB pathway

Negative effectors interfere with the strength and the duration of signalling pathways in a defined window of time, thereby fine-tuning the signal transmission [21]. In the NF-κB pathway, transient inhibition of ubiquitin signals is mediated via the action of DUBs and inhibitory ubiquitin-binding proteins. To date, two DUBs have been identified to have important roles in regulating the NF-κB pathway: A20 and CYLD (cylindromatosis) (Figure 1). A20 has a dual role in down-regulating NF-κB signalling. First, A20 specifically removes Lys^{63}-linked ubiquitin chains from RIP, an essential mediator of TNFR1 signalling, and subsequently it attaches Lys^{48}-linked ubiquitin chains to promote its proteasomal degradation [22]. CYLD inhibits NF-κB activation by cleaving Lys^{63}-linked polyubiquitin chains from different

proteins such as TRAF-2, TRAF-6 and NEMO [23]. *Cyld* is a tumour-suppressor gene mutated in familial CYLD, also called 'turban tumour syndrome', an autosomal dominant predisposition to multiple tumours of the skin appendages [24]. A recent study showed that CYLD also de-ubiquitinates the transcriptional co-activator Bcl-3 and prevents its nuclear translocation where it would collaborate with p50 and p52 proteins. In this way, CYLD action leads to suppression of transcription of the NF-κB target genes. Moreover, *Cyld*-deficient mice had no defect in the transcription induced by the classical p65–p50 NF-κB dimers, but only showed excessive NF-κB activation via Bcl-3-linked p50- or p52-dependent gene regulation, which contributes to the high sensitivity to skin tumour development in these mice [25]. Interestingly, a recent report described a new regulatory role for CYLD in TCR signalling. *Cyld*-deficient mice are impaired in thymocyte development due to aberrations in the transition of double-positive to single-positive T-cells. In contrast with the finding in keratinocytes, there are no defects in TNFR-induced NF-κB signalling in BMDMs (bone-marrow-derived macrophages) of *Cyld*-deficient mice. The T-cell phenotype of *Cyld*-deficient mice resembles the ZAP-70 [ζ-chain (TCR)-associated protein kinase of 70 kDa]-deficient thymic phenotype and it was postulated that CYLD positively regulates TCR signalling in thymocytes by promoting the recruitment of active Lck to its substrate, ZAP-70. The role of CYLD in regulation of Lck ubiquitination and its impact on TCR signalling is unclear at present.

ABIN (A20-binding inhibitor of NF-κB) proteins compose a family of cytosolic proteins (ABIN-1, -2 and -3), which function in a collaborative manner with A20 to inhibit NF-κB signalling. ABIN-1 was identified as an interactor of A20 and binds to the C-terminus of A20 [26]. Moreover, it was shown that the NF-κB-inhibiting function of A20 can be mimicked by overexpression of ABIN proteins and may even be partially mediated through ABIN-1, ABIN-2 and ABIN-3 [27]. ABIN proteins share a number of conserved regions termed AHD1–3 (ABIN homology domains 1–3). Interestingly, ABIN was found to possess a UBD that is similar to the one present in NEMO and Optineurin. Deletion of this conserved region resulted in the loss of ABIN's inhibitory functions [28–30]. Although the ABINs have not yet been confirmed to be *bona fide* ubiquitin-binding proteins, the current indications lead to the exciting possibility that their ability to bind to ubiquitinated components of the NF-κB pathway may be central to their role as negative regulators.

Conclusion

Multiple signalling mechanisms, including allosteric changes, compartmentalization of activators or effectors and post-translational modifications, contribute to the regulation of NF-κB signalling pathways. Among these mechanisms, ubiquitination acts as a signalling device, fine-tuning and regulating cellular signalling networks. The complete knowledge of the signalling interplay between ubiquitination and other post-translational modifications will have a great impact on our understanding of biological processes and contribute to our ability to design novel tools able to manipulate these processes for therapeutic purposes.

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