Molecular Cancer

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Hypothesis

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At the crossroads of SUMO and NF-κB

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Published: 05 November 2003

Molecular Cancer 2003, **2**:39

Received: 01 September 2003

Accepted: 05 November 2003

This article is available from: http://www.molecular-cancer.com/content/2/1/39

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Abstract

Background: Recognition of pathogens by immune receptors leads to activation of macrophages, dendritic cells, and lymphocytes. Signals are communicated to enhance expression of target molecules such as cytokines and adhesion molecules, depending on activation of various inducible transcription factors, among which the family NF-κB transcription factors plays an evolutionarily conserved and critical role. Classical activation of NF-κB involves phosphorylation, polyubiquitination and subsequent degradation of the inhibitor molecules of NF-κB, referred to as IκB. Modification of IκBα, one of the mammalian IκB isoforms, with the small ubiquitin-like modifier (SUMO) results its protection from degradation.

Presentation of the hypothesis: SUMO-IκBα localizes in the nucleus. The nuclear SUMO-IκBα pool may be dynamic. SUMO-IκBα functions as synergy control factor.

Testing the hypothesis: Immunoprecipitation from cellular fractions, ^{35}S methionine pulse-chase, and FRET assays should reveal the localization of SUMO-IκBα and the dynamics of the pool. Expression of SUMOylation defective IκBα in an *IκBα* $^{-/-}$ background should yield insights into the function of SUMO-IκBα.

Implication of the hypothesis: IκBα contains the required SUMOylation motif but IκBβ does not. The suggested study would provide evidence whether or not IκBα and IκBβ can substitute each other. In addition, the suggested assays would reveal a possible redundancy in controlling transcriptional activity of NF-κB.

Background

For nearly two decades, the NF-κB activation pathway has been the focus of experimental investigation. This pathway is highly conserved among metazoans and plays key roles in immune responses [1], cell proliferation [2], inflammation [3], apoptosis [4], early embryonic development [5], and many other processes.

Five members of mammalian NF-κB are described: NF-$κB_1$ (p50 and its precursor p105), NF-$κB_2$ (p52 and its precursor p100), c-Rel, RelA (p65) and RelB [6–9], each of which has a 300-residue Rel homology domain (RHD) [1,10–14]. The C-terminal domains are responsible for dimerization with other Rel proteins, but sequence-spe-

cific interactions come primarily from loops in the N-terminal domain [15].

Interaction of c-Rel, RelA and RelB with its inhibitors, referred to as IκB, results in inactive complexes in the cytoplasm by masking the nuclear localization signal, which is located at the C terminal end of the Rel homology domain [1,10–14]. Currently, the inhibitor proteins IκBα, IκBβ, IκBγ, IκBε, Bcl-3, and the Drosophila protein Cactus are described and characterized [1,10–14].

In most cell types, NF-κB proteins are sequestered in the cytoplasm in an inactive form through their non-covalent association with the inhibitor IκB [7]. This association masks the nuclear localization signal of NF-κB, thereby preventing NF-κB nuclear translocation and DNA binding activity [16]. NF-κB is activated through complex signaling cascades that are integrated by activation of IκB kinase complex (IKK) [17–21], which phosphorylates IκB bound to NF-κB complexes as its substrates [22]. Consequently, NF-κB proteins are translocated into the nucleus, where they activate transcription of their target genes [11,12]. One of the key target genes regulated by NF-κB is its inhibitor IκBα. A feedback inhibition pathway for control of IκBα gene transcription and down-regulation of transient activation of NF-κB activity is described [23–25].

Signal-induced degradation of IκB family members has traditionally been considered to regulate NF-κB activity. However, accumulating evidence suggests that NF-κB activity is also controlled by transcriptional repression mediated by Rel-family members [26], phosphorylation of RelA [27–29], NF-κB_2 [30], and interaction of RelA and NF-κB_2 with histone deacetylases (HDACs) [31–33]. In addition, modification of *Dorsal* by *Smt3*, the *Drosophila melanogaster* orthologs of NF-κB and SUMO-1, respectively, has been demonstrated to regulate *Dorsal*-mediated activation [34]. Indeed, modification of proteins at lysine residues by ubiquitination, SUMOylation and acetylation has been suggested as a means by which control of transcriptional responses in general can be fine-tuned [35].

Small ubiquitin-like modifier (SUMO) is the best-characterized member of a growing family of ubiquitin-related proteins. SUMO is conjugated to target proteins using an enzyme conjugation system similar to that of ubiquitin [36,37]. The targets of SUMOylation suggest roles for protein SUMOylation in TNFR-mediated apoptosis [38] and, paradoxically, protection from cell death [39]; nucleocytoplasmic transport [40–44] and subnuclear [45] localization; and modulation of transcription factor activity [46,47].

Among the perhaps more intriguing targets for SUMO modification is IκBα. Polyubiquitination of IκBα chiefly at lysine residues 21 and 22 targets it for proteasomal degradation, whereas SUMOylation at lysine 21 appears to protect IκBα from proteasome-mediated degradation [48]. Furthermore, current data suggest that SUMOylation of IκBα is an event that occurs in the nucleus, as pyruvate kinase-IκBα fusion protein constructs that lack a functional NLS fail to be modified by SUMO [49].

Presentation of the hypothesis

Desterro et al showed that a modified form of IκBα is found in whole cell extracts from HEK 293, COS7, Jurkat, and HeLa cell lines. Reciprocal immunoprecipitation experiments with anti-IκBα and anti-SUMO-1 antibodies demonstrated that IκBα is SUMOylated. Further experimentation revealed that the level of SUMO-IκBα in whole cell lysates from TNFα-stimulated COS7 cells remains constant over time, whereas levels of unmodified IκBα diminish and are replenished over time, consistent with a previous study [23].

In parallel, cellular fractionation was carried out on these cell lines. Cytoplasmic extracts showed little to no SUMO-IκBα. Roff et al suggest that SUMO-IκBα is located in the nucleus [50]. Supporting these data are data by Rodriguez et al that suggest that IκBα retained in the nucleus after LMB treatment is impervious to signal-induced degradation [52].

We therefore hypothesize that SUMO-IκBα localizes in the nucleus, creating a nuclear pool of SUMO-IκBα, which functions as synergy control factor.

Testing the hypothesis

Data from reporter gene assays suggest a role for SUMO-IκBα in inhibiting NF-κB-dependent transcription [48]. What may however be complicating assessment of function is the fact that the reporter gene data derive from cells in which SUMO is overexpressed. Given that little free SUMO-1 is detectable in mammalian cells [53], and that other members of the NF-κB pathway can crosstalk with Ubc9, the SUMO E2 conjugating enzyme [54], overexpression of SUMO-1 may result in phenotypes that do not correspond to in vivo situations. Moreover, all extant data regarding SUMO-IκBα stem from transformed and/or oncogenic cell lines, in which NF-κB activity cannot necessarily be assumed normal. To date, no data show the existence of SUMO-IκBα in primary cell lines. The following experiments are proposed in order to assess the presence of SUMO-IκBα in primary cells, and the physiological relevance of SUMO-IκBα.

Dynamics of the SUMO-IκBα pool

Hay et al stated that levels ranging from barely detectable to almost 50% of the total IκBα can be found in the cell types examined in their experiments [48,55]. The cell lines

investigated were HEK 293, COS7, Jurkat and HeLa. In order to establish the extent of IκBα SUMOylation in non-transformed/non-oncogenic cell lines, the following experiments might prove to be of considerable interest, and should not entail significant technical difficulties. From wild-type mice, isolate fibroblast and B- and T-cell populations. These primary cells could be directly used in the subsequent experiments. However, strategies for creating genetically stable, nontumorigenic immortalized cell lines have been described [56,57]. The use of these or comparable strategies for creating immortalized, genetically stable and nontumorigenic fibroblast, B- and T-cell populations may therefore be useful for keeping these primary cells in culture for repeat experiments. With these cell lines in hand, prepare cytoplasmic and nuclear fraction of cells according to Roff et al [50], and whole cell extracts as described by Desterro et al [48]. Reserve some of the whole cell extracts for immunoprecipitation experiments. Fractionate the cytoplasmic, nuclear and whole cell extracts on a denaturing, reducing polyacrylamide gel and transfer to membranes for immunoblot analysis with an anti-IκBα antibody. In parallel, immunprecipitate with anti-SUMO-1 antibody as described [58], followed by an IκBα Western analysis, and vice versa [48].

Fluorescence resonance energy transfer (FRET) may present an additional means by which a nuclear association of SUMO-1 and IκBα could be demonstrated. CFP-IκBα and SUMO-YFP have both been described [47,59], as has the use of FRET microscopy to assess protein interactions in living cell nuclei [60]. As stated previously, overexpression experiments are potentially problematic due both to interactions and localization that may occur only when proteins are overexpressed. Therefore, the use of a targeted knock-out/knock-in strategy to make CFP-IκBα and SUMO-YFP double mutant mice might provide the specificity needed in this experimental system. The creation of such mice may be time consuming, yet the techniques for making the mice are established.

Two different options suggest potential (and mutually inclusive) roles for the pool in controlling NF-κB responses and in nucleocytoplasmic transport. To address the issue of static vs. dynamic pools of SUMO-IκBα, the following experiment is suggested: A pulse-chase ^{35}S-methionine assay using cytoplasmic, nuclear and whole extacts from cells at time points described above and by Desterro et al [48,61].

Both nucleocytoplasmic trafficking and nuclear translation [62] would result in radiolabeled proteins being found in the nucleus. However, most of these proteins are not expected to associate with IκBα pulled down in the immunoprecipitation. If, contrary to expectation, radiolabeled proteins other than Rel family members are found to associate with IκBα, the likelihood that any of these would have the same molecular weight as SUMO-IκBα is assumed to be manageably small. If no radiolabel is detected at 36 kD, this suggests that the SUMO-IκBα pool does not turn over appreciably. If radiolabel is detected at 36 kDa, this would suggest that, while the total pool of SUMO-IκBα remains constant, this pool turns over. Changing levels of radioactivity would suggest that SUMO-IκBα turns over in the nucleus. This in turn may indicate a key role in nucleocytoplasmic transport to ensure a constant nuclear pool of IκBα.

SUMO-IκBα – a Synergy Control Factor

Cheng et al created IκBα/IκBβ knockout/knock-in mice, and no abnormalities in viability or reproduction were observed in these animals [63]. This suggests a functional redundancy of these two IκB family members. However, IκBα is rapidly degraded upon stimuli that trigger NF-κB responses, whereas IκBβ is degraded at a significantly slower rate [64]. In addition, the observation that deletion of IκBα or IκBβ from the genome appears to be compensated by other IκB family members [65,66], but that deletion of IκBα results in neonatal lethality [67], together hint at distinct roles for IκBα and IκBβ in NF-κB responses. Inspection of the amino acid sequence of IκBβ shows it lacks the Ψ KXE amino acid motif characteristic of proteins that are SUMOylated. Therefore, IκBα/IκBβ knockout/knock-in mice may phenocopy mice in which wild-type IκBα is replaced with IκBα^{K21R}, a mutant IκBα for which SUMOylation, but not ubiquitination, is compromised.

The creation of IκBα/IκBβ knockout/knock-in mice indicates that the creation of IκBα^{K21R} mice is feasible. Whether or not such mutant mice phenocopy IκBα/IκBβ knockout/knock-in mice, the viability of the IκBα/IκBβ mouse suggests that IκBα^{K21R} mutants would also live to reproductive age. The following suggested experiments should prove to be informative with respect to SUMO-IκBα function: Create IκBα/IκBα^{K21R} mutant mice using established protocols [68,69]. Assess viability. Challenge immune systems of mutant, wild-type and IκBα/IκBβ knockout/knock-in mice as described [70,71], and assess immune responses at the level of the organism. Establish immortalized cell lines from the IκBα/IκBα^{K21R} mutant mice as described above. Stimulate an NF-κB response using TNF-α in fibroblasts and lymphoid immortalized cell lines from IκBα^{K21R} mutant, wild type, and IκBα/IκBβ knockout/knock-in mice and analyze the response.

The Ψ KXE amino acid sequence element required for SUMOylation strongly resembles what is referred to as a synergy control (SC) motif. SC motifs in certain transcription factors (TFs) have been shown to confer onto TFs the property of downregulating transcription at promoters

with multiple binding sites for that TF, while promoters with single TF binding sites are unaffected. When the SC motifs are disrupted, transcription from promoters with multiple sites is enhanced. SUMOylation of TFs at SC motifs has been demonstrated in at least two cases [34,72], suggesting role for SUMOylation in transcriptional control.

Does SUMOylation of IκBα play a role in transcriptional control? A nuclear pool of SUMO-IκBα may help titrate NF-κB and influence transcriptional responses. As stated previously, NF-κB transcriptional responses can be regulated at the level of repression by certain Rel-family homodimers [26], by phosphorylation [27–29], and by interaction with histone deacetylases [31–33]. Therefore, titration of NF-κB by nuclear IκBα, whether SUMOylated or not, may be a redundant mechanism to control NF-κB transcriptional activity. Given the general importance of the NF-κB pathway, redundancy in controlling transcriptional responses by ensuring a nuclear pool of IκBα may be useful.

Implications of the hypothesis

IκBα contains the required SUMOylation but IκBβ does not. The suggested study may demonstrate whether or not IκBα and IκBβ can substitute each other. In addition, the suggested assays would reveal a possible redundancy in controlling transcriptional activity of NF-κB.

Authors' contributions

MPK drafted the paper. CS provided suggestions for its finalization. Both authors read and approved the final manuscript.

Acknowledgements

CS acknowledges support from the German Research Foundation.

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