Title
A Regulated, Ubiquitin-Independent Degron in IκBα.

Permalink
https://escholarship.org/uc/item/3r37s7p3

Journal
Journal of molecular biology, 427(17)

ISSN
0022-2836

Authors
Fortmann, Karen T
Lewis, Russell D
Ngo, Kim A
et al.

Publication Date
2015-08-01

DOI
10.1016/j.jmb.2015.07.008

Peer reviewed
Whereas ubiquitin-dependent degrons have been characterized in some detail, how proteins may be targeted to ubiquitin-independent proteasomal degradation remains unclear. Here we show that IκBα contains an ubiquitin-independent degron whose activity is portable to heterologous proteins such as the globular protein GFP (green fluorescent protein) via a proteasome-dependent, ubiquitin-independent, non-lysosomal pathway. The ubiquitin-independent degradation signal resides in an 11-amino-acid sequence, which is not only sufficient but also required for IκBα’s short half-life. Finally, we show that this degron’s activity is regulated by the interaction with NFκB, which controls its solvent exposure, and we demonstrate that this regulation of the degron’s activity is critical for IκBα’s signaling functions.

© 2015 Elsevier Ltd. All rights reserved.
fluorescence. These studies demonstrated that the short half-life control of IκBα could be transferred to the globular protein GFP and that proteolysis was sensitive to proteasome inhibitor.

There are two well-known intracellular protein degradation pathways in mammalian cells: the lysosomal pathway, which is generally non-selective, and the ubiquitin-proteasome system, which is selective via

![Figure 1](legend on next page)
ubiquitin E3 ligases [1]. However, both may be inhibited by MG132 [18]. Pretreating IkBα-expressing nfkβ−/− cells with the lysosomal inhibitors Bafilomycin-A and Chloroquine before administering cycloheximide (CHX) in a time course, we did not observe any decrease in IkBα turnover (Fig. 1b). Only with the MG132 control did we see a rapid increase in protein levels, confirming in vitro studies that free IkBα degradation is mediated by the proteasome [8,10].

To identify the molecular determinants of IkBα’s short half-life, we engineered a number of recombinant forms respecting IkBα’s key structural elements (Fig. 1c). We sought to determine if the C-terminal portion of IkBα shown to be required for short IkBα half-life [8] was a sufficient determinant for degradation and thus fused it to GFP, expressing the resulting GFP-IkBα (210-317) in nfkβ−/− cells. To measure the approximate half-life, we again used cycloheximide time courses and immunoblotted for IkBα. Whereas the GFP control construct was stable during the 60-min time course, the fusion protein showed a half-life of less than 15 min (Fig. 1d). The C-terminal PEST sequence had been suspected to play a role in IkBα turnover. However, we found that the PEST domain of IkBα was not sufficient to grant degradation and in fact was stable during a 60-min time course (Fig. 1d).

It was previously shown that the lysines of free IkBα are not required for free IkBα degradation [8]. However, ubiquitin conjugations on other amino acids such as cysteine, serine, and threonine via thioester and hydroxyester linkages have been reported to mediate proteasomal degradation [19]. To examine whether these atypical ubiquitin acceptors might play a role in the degradation of IkBα, we first assayed for ubiquitin modification using a sensitive assay of overexpressed HA-ubiquitin in HEK293T cells; we found that the GFP-AR56PEST IkBα construct showed no more than GFP background signals unlike the full-length IkBα construct (Fig. S1). We then mutated all lysines, cysteines, serines, and threonines in ankyrin repeats 5 and/or 6 within this construct to alanine (lysine was mutated to arginine) and found that, following retroviral transduction into nfkβ−/− cells, cycloheximide time courses revealed no change in the half-life of the mutants (Fig. 1e).

In sum, our results confirm and extend previous studies [8,10] indicating that the IkBα C-terminus contains a degron, a peptide sequence (other than PEST) that confers ubiquitin-independent degradation by the proteasome.

An 11-amino-acid degron within ankyrin repeat 6

To identify the peptide sequence conferring degradation of IkBα, we generated mutated variants of the fifth and sixth ankyrin repeats (AR5 and AR6) using the GFP fusion expressing nfkβ−/− cell system. In cycloheximide time courses (Fig. 2a), GFP-IkBα (AR5: 210-241) showed very similar degradation kinetics to the stable GFP control and PEST domain of IkBα (Fig. 1c). However, the GFP-IkBα (AR6: 243-280) construct showed rapid degradation analogous to the degradation of the complete C-terminus of IkBα (281-317). The quantitated data showed that the half-life of GFP-IkBα (281-317) and that of GFP-AR5 IkBα (210-241) had half-lives greater than 2 h while the GFP-IkBα (AR6: 243-280) had a...
half-life of around 15 min. These findings indicate that the degron of IκBα is in its sixth ankyrin repeat.

Previous work established that the short half-life of thymidine synthase depends on an ubiquitin-independent degron, which must be located at its very N-terminus, as blocking the N-terminus with a His tag caused protein stabilization [20]. To examine whether the IκBα degron function depended on its C-terminal location, we engineered a construct in which the HA-GFP tag was fused to the C-terminus rather than to the N-terminus of the 243-280 region (AR6) (Fig. 2b). Cycloheximide time course data show that the degron’s location does not affect its activity. The C-terminal region of IκBα is known to be incompletely folded when not bound to NFκB [9,21]. We asked whether the internal ankyrin repeats AR2 and AR3 may be harboring latent degrons also, hidden within the folded ARD of AR1–AR4. As three ARs are required for stable folding of an ARD [22], we examined AR2 and AR3 in isolation but neither

Fig. 2. The degron resides in ankyrin repeat 6. (a) Immunoblot for HA with antibody 16b12 (Covance), followed by HRP conjugate, of whole cell extracts prepared from nfkβ−/− cells expressing AR5 [IκBα (210-241)] or AR6 [IκBα (242-280)] treated with CHX, as previously described. Right panel shows quantification of experiments, and error bars indicate standard deviation representative of three experiments. (b) Immunoblot for HA of whole cell extracts prepared from nfkβ−/− cells expressing AR6 [IκBα (242-280)] with the HA-GFP tag at either the N-terminus or the C-terminus of the protein, treated with CHX. Right panel shows quantification of experiments, and error bars indicate standard deviation and are representative of at least three experiments. (c) Immunoblot for HA of whole cell extracts prepared from nfkβ−/− cells expressing AR3 [IκBα (137-176)] or the AR2 [IκBα (104-136)] treated with CHX. Right panel shows quantification of three experiments, and error bars indicate standard deviation.
A Regulated, Ubiquitin-Independent Degron in IκBα

**Fig. 3 (legend on next page)**
caused similar degradation of GFP after a 60-min exposure to CHX (Fig. 2c). These results indicate that there are specific degron sequences within AR6 that are not present in other ARs of IkBα.

To determine in more detail which segment of AR6 is responsible for free IkBα degradation, we exploited the functional differences between the structurally homologous AR3 and AR6 and generated chimeric constructs (Fig. 3a). First, we fused the first half of AR6 to the second half of AR3 (mutant 6633) and vice versa (mutant 3366). Within the \textit{nkb}^{−/−} cell system, the 3366 mutant showed a half-life greater than 60 min while the 6633 mutant had a half-life of 28 min similar to the full ankyrin repeat 6 (Fig. 3b). These results suggested that the first half of AR6 (IkBα 243-262) contains the IkBα degron activity.

In order to further identify the amino acid sequence responsible for IkBα degradation, we integrated either the first or the second quarter of AR6 into AR3 (mutant 6333 or 3633; Fig. 3a). Within the \textit{nkb}^{−/−} cell system, we observed a relatively short half-life for the 3633 mutant (Fig. 3b) while the 6333 mutant was more stable. These results show that primary IkBα degron activity is mediated by residues 251–262. We made further attempts to locate specific amino acids critical for its activity. Given that 3 of the 11 residues are aromatics, we wondered whether they may be involved in ubiquitin-independent proteasomal targeting. However, mutating Y251A, Y254A, and W258A, singly or in combination, did not result in a longer half-life (Fig. S2). As the 3633 mutant did not show a half-life as short as the full-length AR6 or the 6633 mutant, we conclude that additional residues within the first half of AR6 may also contribute to the degron's activity.

Our studies thus far identified the first half of ankyrin repeat 6 of IkBα as sufficient for triggering degradation of the heterologous reporter protein GFP. Now we asked whether this sequence might also be required for IkBα degradation in the context of the full-length protein. We engineered a mutant form of IkBα that had its sixth ankyrin repeat replaced with the 3366 chimeric repeat (Fig. 3c), stably transduced this construct, as well as wild-type IkBα and the long-lived IkBα (1-206) controls into \textit{nkb}^{−/−} cells, and treated these cell lines with cycloheximide. Immunoblotting for IkBα revealed a stabilization of IkBα in the 36Mut that was similar to the IkBα (1-206) control (Fig. 3d). These results demonstrate that the first half of AR6 contains a degron sequence that is both sufficient for degrading heterologous proteins and required for the degradation of free IkBα. We conclude that the degron of IkBα has a non-redundant function in IkBα degradation.

The degron must be regulated to provide for proper control of NFκB signaling

The degron peptide sequence is located within the previously characterized incompletely folded C-terminus of IkBα [21], and deviations from the AR consensus were noted. Indeed, mutations Y254L and T257A toward the AR consensus increased IkBα foldedness and \textit{in vivo} half-life [9]. Interestingly, testing these mutations within the isolated AR6 in our system did not prolong the protein half-life (Fig. 3d), supporting the notion that these mutations did not delete the degron itself but decreased its activity by increasing the region's foldedness. Similarly, the degron's solvent exposure is obscured when IkBα is bound to NFκB [21,23] (Fig. 3e), correlating with a long half-life [11,14,24].

We asked whether regulating the degron's activity is in fact important for NFκB signaling. To this end, we pursued a synthetic biology approach based on the IkB family member, IkBδ (Fig. 4a). This IkB is fully folded in solution, has a long half-life, and binds RelA:p50 with comparable affinity [25,26]. However, after attaching the IkBα signal-responsive domain SRD to the N-terminus and the IkBα AR6 degron to the C-terminus of IkBδ, the resulting chimera was destabilized in cells not only deficient in NFκB but also—unlike IkBα—containing NFκB (Fig. 4b).

Using an established kinetic model of the IkBα-NFκB signaling module [14,27], we simulated TNF-induced NFκB activity mediated by wild-type IkBα or this mutant whose short half-life was identical whether free or bound with NFκB (Fig. 4c). When the

---

**Fig. 3** An 11-amino-acid sequence is sufficient and required. (a) Schematic detailing the amino acids in AR3 (in gray) and AR6 (in pink). The four chimeras (3366, 6633, 3633, and 6333) contain the indicated amino acids derived from the color-coded AR. (b) Immunoblot for HA of whole cell extracts prepared from \textit{nkb}^{−/−} cells expressing WT IkBα, IkBα 36Mut, or IkBα (1-206) treated with CHX. Bottom panel shows quantification of experiments, and error bars indicate standard deviation. (c) The degron has a non-redundant role in IkBα degradation. Top, schematic of the 36Mut IkBα. Bottom, immunoblot for the N-terminus of IkBα of \textit{nkb}^{−/−} cells expressing WT IkBα, IkBα 36Mut, or IkBα (1-206) treated with CHX. Right panel shows quantifications of three experiments, and error bars indicate standard deviation. (d) The degron does not rely on Y254 or T257, previously identified as maintaining the unfolded state (REF). Immunoblot for IkBα of whole cell extracts from \textit{nkb}^{−/−} cells transduced with either GFP-IkBα (210-317) YLTA (whereby Y254 was mutated to leucine and T257 was mutated to alanine) or GFP-IkBα (210-317) treated with CHX for indicated times. (e) Location of the degron in the IkBα-NFκB complex. A ribbon diagram representation of the crystal structure of IkBα (pink) bound to NFκB (p50, cyan; p65, green). The location of the 11-amino-acid degron in black, depicted in a ball-and-stick representation. The figure was prepared using the PyMOL Molecular Graphics System, Version 1.7 Schrödinger, LLC.
half-lives were long, little NFκB activation was seen as the resulting IkB overexpression functioned as a transdominant inhibitor; when the half-lives were short, signaling was recovered; however, the basal NFκB activity was predicted to be elevated.

Using these IkB variants, we examined their effects on TNF-inducible NFκB activity. As expected, the fully stable IkB functioned as a transdominant inhibitor to suppress NFκB activation, compared to the IkBα positive control. In contrast, the destabilized chimera did allow for NFκB signaling, but we noted an elevated basal level of NFκB activity (Fig. 4d), as predicted. These results support the notion that NFκB control of IkBα half-life is critical for proper regulation of NFκB activity under basal and stimulus-induced conditions.

We then explored the effect of an unregulated degron on NFκB signaling dynamics that are largely shaped by the NFκB-IkBα negative feedback loop. Kinetic model simulations predicted that, while the IkBα harboring a deregulated degron may provide some degree of post-induction attenuation, the extent and timing is defective, such that oscillations normally seen with wild-type IkBα cannot be
sustained (Fig. 4e). We tested these predictions with a retroviral construct that expresses the IκB variants under the control of five κB sites. Indeed, even with NFκB feedback, the unregulated degron does not recapitulate IκB’s function (Fig. 4f). These data suggest that NFκB control of the IκB degron is essential for achieving the characteristically oscillatory NFκB signaling dynamics.

In this study, we have reported the identification and characterization of a signal sequence that triggers the ubiquitin-independent, proteasome-dependent degradation of IκBα. Our work clarifies that the so-called PEST region of IκBα is not a degradation signal for IκBα (although PEST sequences are generally thought to be responsible for protein turnover [28]) and that the lack of foldedness and solvent exposure is required but not sufficient for ubiquitin-independent degradation. Indeed, the identification of a specific sequence suggests new avenues for studying the biophysical basis of ubiquitin-independent degradation by the proteasome.

Acknowledgements

We thank E. Komives, G. Ghosh, and J. Dyson for their critical advice and support throughout this project. K.T.F. acknowledges J. A. Vargas for microscopy training and support from an Interfaces Training Grant, and A.H. acknowledges support from National Institutes of Health grants R01 GM071573, R01 CA141722, and P01 GM071862.

Author Contributions: A.H. and K.T.F. designed experiments, K.T.F. generated and analyzed the experimental data, assisted by R.L. and K.A.N. R.F. generated the inducible construct. A.H. and K.T.F. wrote the manuscript.

Conflict of Interest: The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2015.07.008.

Received 23 October 2014; Received in revised form 12 June 2015; Accepted 13 July 2015
Available online 17 July 2015

Keywords:
protein half-life control; degron; ubiquitin-independent degradation; NFκB; IκBα

References