A Regulated, Ubiquitin-Independent Degron in IκBα

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Abstract

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.

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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

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

To identify the molecular determinants of IkBa’s short half-life, we engineered a number of recombinant forms respecting IkBa’s key structural elements (Fig. 1c). We sought to determine if the C-terminal portion of IkBa shown to be required for short IkBa half-life [8] was a sufficient determinant for degradation and thus fused it to GFP, expressing the resulting GFP-IkBα (210-317) in nktb−/− cells. To measure the approximate half-life, we again used cycloheximide time courses and immunoblotted for IkBa. 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 IkBa turnover. However, we found that the PEST domain of IkBa 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 IkBa are not required for free IkBa degradation [8]. However, ubiquitin conjugations on other amino acids such as cysteine, serine, and threonine via thiodiester and hydroxyster linkage have been reported to mediate proteasomal degradation [19]. To examine whether these atypical ubiquitin acceptors might play a role in the degradation of IkBa, we first assayed for ubiquitin modification using a sensitive assay of overexpressed HA-ubiquitin in HEK293T cells; we found that the GFP-AR56PEST IkBa construct showed no more than GFP background signals unlike the full-length IkBa 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 nktb−/− 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 IkBa 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 IkBa, we generated mutated variants of the fifth and sixth ankyrin repeats (AR5 and AR6) using the GFP fusion expressing nktb−/− 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 IkBa (Fig. 1c). However, the GFP-IkBα (AR6: 243-280) construct showed rapid degradation analogous to the degradation of the complete C-terminus of IkBa (281-317). The quantitated data showed that the half-life of GFP-IkBα (281-317) and that of GFP-AR5 IkBa (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 IkBa 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 IkBa 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 IkBa is known to be incompletely folded when not bound to NFkB [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

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**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 nfkb−/− cells expressing AR5 [IkBa (210-241)] or AR6 [IkBa (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 nfkb−/− cells expressing AR6 [IkBa (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 nfkb−/− cells expressing AR3 [IkBa (137-176)] or the AR2 [IkBa (104-136)] treated with CHX. Right panel shows quantification of three experiments, and error bars indicate standard deviation.
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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 IκBα.

To determine in more detail which segment of AR6 is responsible for free IκBα 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 nfkβ−/− 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 half-life for the 3633 mutant (Fig. 3b) while the 6333 mutant was more stable. These results show that the primary IκBα 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 6333 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 IκBα as sufficient for triggering degradation of the heterologous reporter protein GFP. Now we asked whether this sequence might also be required for IκBα degradation in the context of the full-length protein. We engineered a mutant form of IκBα that had its sixth ankyrin repeat replaced with the 3366 chimeric repeat (Fig. 3c), stably transduced this construct, as well as wild-type IκBα and the long-lived IκBα (1-206) controls into nfkβ−/− cells, and treated these cell lines with cycloheximide. Immunoblotting for IκBα revealed a stabilization of IκBα in the 36Mut that was similar to the IκBα (1-206) control (Fig. 3c). 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 IκBα. We conclude that the degron of IκBα has a non-redundant function in IκBα 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 IκBα [21], and deviations from the AR consensus were noted. Indeed, mutations Y254L and T257A toward the AR consensus increased IκBα foldedness and 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 IκBα 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 IκB family member, IκBδ (Fig. 4a). This IκB is fully folded in solution, has a long half-life, and binds RelA:p50 with comparable affinity [25,26]. However, after attaching the IκBα signal-responsive domain SRD to the N-terminus and the IκBα AR6 degron to the C-terminus of IκBδ, the resulting chimera was destabilized in cells not only deficient in NFκB but also—unlike IκBα—containing NFκB (Fig. 4b).

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

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**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 nfkβ−/− cells expressing the four chimeras treated with CHX. Bottom panel shows quantification of experiments, and error bars indicate standard deviation. (c) The degron has a non-redundant role in IκBα degradation. Top, schematic of the 36Mut IκBα. Bottom, immunoblot for the N-terminus of IκBα of nfkβ−/− cells expressing WT IκBα, IκBα 36Mut, or IκBα (1-206) treated with CHX. Right panel shows quantification of 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 IκBα of whole cell extracts from nfkβ−/− cells transduced with either GFP-IκBα (210-317) YLTA (whereby Y254 was mutated to leucine and T257 was mutated to alanine) or GFP-IκBα (210-317) treated with CHX for indicated times. (e) Location of the degron in the IκBα-NFκB complex. A ribbon diagram representation of the crystal structure of IκBα (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 NFkB 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 NFkB activity was predicted to be elevated.

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

We then explored the effect of an unregulated degron on NFkB signaling dynamics that are largely shaped by the NFkB-IkB negative feedback loop. Kinetic model simulations predicted that, while the IkB 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.

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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.

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