Mechanism of direct degradation of IκBα by 20S proteasome

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Abstract IκBα regulates activation of the transcription factor NF-κB. NF-κB is activated in response to several stimuli, i.e. proinflamatory cytokines, infections, and physical stress. This signal dependent pathway involves IκBα phosphorylation, ubiquitylation, and degradation by 26S proteasome. A signal independent (basal) turnover of IκBα has also been described. Here, we show that IκBα can be directly degraded by 20S proteasomes. Deletion constructs of IκBα allow us to the determine that N-terminal (ΔN 1–70) and C-terminal regions (ΔC 280–327, removing the PEST region) of IκBα are not required for IκBα degradation, while a further C-terminal deletion including part of the arm repeats (ΔC2 245–327) almost completely suppress the degradation by 20S proteasome. Binding and competition experiments demonstrate that the degradation of IκBα involves specific interactions with α2(C3) subunit of the proteasome. Finally, p65/relA (not itself a substrate for 20S proteasome) inhibits the degradation of IκBα by the proteasome. These results recapitulate in vitro the main characteristics of signal independent (basal) turnover of IκBα demonstrated in intact cells.

Keywords: IκBα; NF-κB; Proteasome; Proteolysis; p65

1. Introduction

The ubiquitin-proteasome system is responsible for the degradation of most part of proteins in eukaryotic cells. This system is involved in a series of cellular functions including: degradation of bulk proteins, cell cycle, cell differentiation, apoptosis, DNA repair, antigen presentation, vesicle transport and regulation of signal transduction pathways and transcription. The widely accepted pathway implies the targeting of proteins to degradation by specific covalent linkage of a polyubiquitin chain to the protein substrate. The multi-ubiquitylated protein can then be degraded by the 26S proteasome [1,2]. The degradation of transcription factors seems to follow this ubiquitin-proteasome pathway. Nevertheless, ubiquitylation of transcription factors in some cases is also required for full activation of the transcription factor [3,4]. Ubiquitylation and limited proteolysis by the proteasome is also implicated in the generation of some active transcription factors: the generation of transcriptionally competent NF-κB isoforms p52 and p50 from their respective precursors p100 and p105 [5,6]; and SPT23 and MGA2, yeast homologues of NF-κB, also require ubiquitylation and proteasome partial proteolytic processing to generate the active transcription factor followed by ubiquitin removal [7,8].

An emerging pathway of protein degradation requires no prior ubiquitylation of the protein substrate for its degradation by the proteasome. A classical example of this pathway is ornithine decarboxylase that is directly degraded by 26S proteasome after binding to antizyme [9], and more recently by 20S proteasome in antizyme independent, NAD(P)H quinone oxidoreductase 1 (NQO1) dependent pathway [10]. Direct degradation by 20S proteasome have also been documented for some proteins with little secondary structure, like: myelin basic protein [11], p21 [12], synuclein [13–15], tau [16,17], or even with tertiary structure like Cot kinase [18]. Some transcription factors have been shown to be degraded by the proteasome without requirement of prior ubiquitylation: IκBα [19–21], c-fos [22], and more recently p53 by NQO1 regulated pathway [23]. Finally, 20S proteasome have also been shown to produce limited proteolysis of GRK2 [24] and initiation factors eIF4G and eIF3a without need of prior ubiquitylation [25].

The classical pathway of signal-induced activation of NF-κB is initiated by activation the IκBα kinases (IKK1 and IKK2) by pro-inflamatory cytokines. The phosphorylation of IκBα at serines 32 and 36 targets IκBα to polyubiquitylation by Ubc5/SCF (beta-TrCP1) and then to degradation by the 26S proteasome [6,26–28]. Apart of this signal dependent pathway of degradation of IκBα, several reports have analyzed the "so-called" signal-independent (basal) pathway of degradation that is also mediated by the proteasome [19–21,29,30]. Here, we show that IκBα can be directly degraded by the 20S proteasome, this degradation is likely to be mediated, at least in part, by interaction with α2(C3) proteasomal subunit and can be negatively modulated by the association of IκBα with p65/RelA. These results recapitulate in vitro the main facts of basal turnover of IκBα in intact cells.

2. Materials and methods

2.1. In vitro coupled transcription–translation

The different constructs were in vitro transcribed with T7 RNA polymerase, and translated in the presence of ^{35}S methionine/cysteine (Amersham) using the TNT system (Promega) according to the manufacturer instructions. IκBα and p65 constructs were provided by Dr. Ulrich Siebenlist (NIAID, National Institutes of Health, Bethesda, MD, USA). Dr. Allan Israel (Institut Pasteur, Paris, France) and Dr. Claus Scheidereit (Max Delbruck Center for Molecular Medicine, Berlin, Germany). Proteasomal alpha subunits were also transcribed and translated in vitro using the TNT system (Promega) from their

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respective cDNA clones (some of these cDNA clones were provided by Dr. Keiji Tanaka, Department of Molecular Oncology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan).

2.2. Protein purification

Rat liver proteasome was purified as described [31,32]. Recombinant GST-IκBα was obtained from induced bacterial cultures, purified on glutathione sepharose and cleaved by thrombin digestion as described [33]. The full length cDNAs encoding for C3, C8, and C9 subunits [11] were subcloned form the pT7-7 vector into the pET15B (*Nde*1/*Sal*1) and the recombinant proteins were purified by Ni-affinity chromatography and dialyzed against 25 mM Tris–Cl, pH 7.5, 50 mM NaCl, insoluble proteins were removed by centrifugation at 100 000 × *g* for 30 min.

2.3. In vitro degradation assays

Degradation reactions contained in a final volume of 20 μl: 20 mM HEPES, pH 7.4, 2 mM EDTA, 1 mM EGTA, 1 μl of the respective ^{35}S-labeled labeled protein (or 0.5 μg of purified recombinant IκBα, 0.7 μM) and 0.5–1 μg of purified rat liver proteasome (35–70 nM). Reactions were incubated at 37 °C for the times indicated and stopped with concentrated SDS–PAGE sample buffer. Control reactions contained 25 μM MG132 or 10 μM lactacystin (proteasome inhibitors), or no proteasome (control for endogenous degradation). Samples after boiling for 5 min were loaded onto 10% SDS–PAGE. Gels were stained, destained, dried under vacuum, and exposed to X-ray film at −70 °C for 14–24 h when required. Quantitation was performed using the Quantity-one software (Biorad). Immunoblot of IκBα degradation were performed as described [34]. Degradation reactions of IκBα in the presence of proteasomal subunits contained 0.4–2 μg (average final concentrations based on 25 000 average molecular mass: 0.8–4 μM) of the indicated recombinant subunit in the degradation mixtures described above.

2.4. Immunoprecipitation assays

In vitro transcribed and translated IκBα (5 μl) was mixed with 10 μl of each of the in vitro transcribed and translated proteasomal alpha subunits in a buffer containing in a final volume of 250 μl: 50 mM Tris–Cl, pH 7.5, 100 mM NaCl, and 0.1% NP40. After centrifugation for 10 min at 14 000 × *g* at 4 °C, to remove any protein aggregates, the mix was incubated for 2 h at room temperature. Anti-IκBα antibodies (5 μl) bound to protein-A Sepharose (Santa Cruz Biotechnology, sc-847) were added and incubation continued for 3 h at 4 °C with shaking. The immunoprecipitates were collected by centrifugation for 10 s at 10 000 × *g* and washed 3 times with 1 ml of 50 mM Tris–Cl, pH 7.5, 100 mM NaCl, and 0.1% NP40. The final pellet was resuspended in SDS–PAGE sample buffer, loaded onto 12% SDS–PAGE and the gels were processed for autoradiogaphy as described above.

3. Results

3.1. Degradation of IκBα by 20S proteasome

Purified recombinant IκBα is directly degraded by 20S proteasome in a time-dependent manner (Fig. 1). This degradation is also dependent on the amount of 20S proteasome and blocked by the presence of 10 μM lactacystin (data not shown). It is worth to note the appearance of transient protein intermediates when the degradation reaction was developed with anti-IκBα antibodies (see also Fig. 1), suggesting an endoproteolytic cleavage of IκBα by the proteasome. Similar results were obtained when IκBα was obtained by in vitro transcription/translation (Fig. 2), and again it was noticed the transient appearance of IκBα intermediates, suggestive of endoproteolytic cleavage. The degradation of in vitro transcribed/translated IκBα was unaffected by ATP removal (addition of 2 units of hexokinase and 10 mM glucose), or by inclusion of an ATP regenerating system (10 mM creatine phosphate and 5 units of creatine phosphokinase), indicating that ATP is not required for degradation in this assay, as it is also the case with the previously shown assay with purified recombinant IκBα (data not shown). Due to the fact that IκBα degradation is modulated by phosphorylation, we studied the degradation of an IκBα construct with two point mutation (Ser 32 and 36 to alanine). This construct is also degraded by the 20S proteasome (Fig. 2), indicating that phosphorylation of those Ser residues are not involved in the in vitro degradation of IκBα. To delineate the region of IκBα required for degradation by 20S proteasome, the degradation of deletion constructs of IκBα were studied. The results presented in Fig. 3 indicate that the N-terminus of IκBα (construct IκBα ΔN 1–70) is not required for degradation. Deletion of the PEST sequence located in the C-terminus (IκBα ΔC 280–327) do not significantly affect the degradation rate of this construct (Fig. 3), while further deletion from the C-terminus (Fig. 3), to include a region of the arm-repeats (arm repeat VI), almost completely suppress the degradation of this IκBα construct (ΔC2 245–327). These in vitro results are in perfect agreement with the results obtained with the same constructs by Krappmann et al. [19] for the basal turnover of IκBα in intact cells.

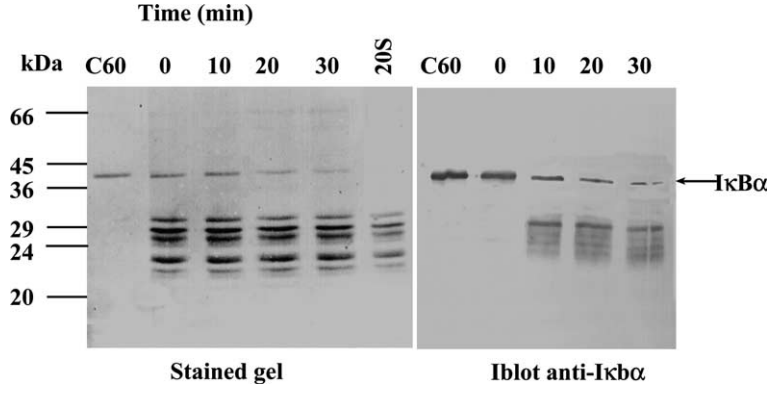

Fig. 1. Time course of the degradation of IκBα by 20S proteasome. Recombinant IκBα 0.5 μg (0.7 μM) was incubated with 1 μg (70 nM) of 20S proteasome for the times indicated and the products analyzed by SDS–PAGE and either directly stained with Coomassie blue (stained gel), or transferred and immunobloted with anti-IκBα antibodies (Iblot). C60, is a control reaction incubated for 60 min without 20S proteasome. 20S lane, is a control lane to display the subunit composition of the 20S proteasome used for the assay.

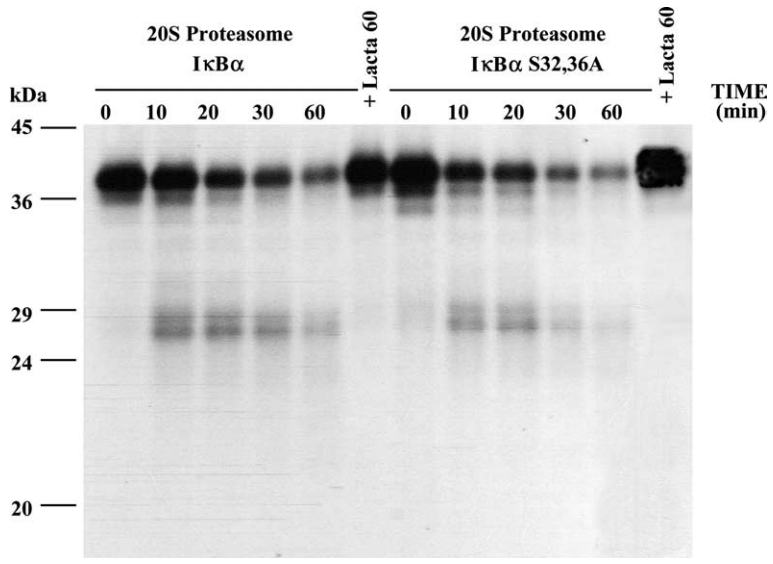

Fig. 2. Time course of the degradation of in vitro transcribed and translated IκBα by 20S proteasome. IκBα wild type and IκBα (S32,36) mutant were in vitro transcribed and translated. Equal amounts of both labeled proteins were incubated for the times indicated and analyzed by SDS–PAGE and autoradiography. Control reactions (Lacta 60) were performed in the presence of lactacystin 10 μM for 60 min.

3.2. Proteasomal subunits involved in the recognition of IκBα

To study the mechanism of recognition of IκBα by the proteasome, we postulate that the initial interaction should take place with proteasomal alpha subunits. To get an insight into which alpha subunit may be involved, we performed in vitro transcription and translation of the 7 different alpha proteasomal subunits and incubate those products with in vitro transcribed and translated IκBα, and the mixtures, after incubation, were subjected to immpunoprecipitation with anti-IκBα antibodies. Fig. 4A shows the products of the in vitro transcription and translation of the 7 alpha proteasomal subunits. Fig. 4B shows the results of the immunoprecipitation experiments, clearly demonstrating that IκBα associates with α2(C3) subunit. If these results are correct, one would expect that recombinant α2(C3) subunit should inhibit the degradation of IκBα. We purified recombinant α2, α3 and α7

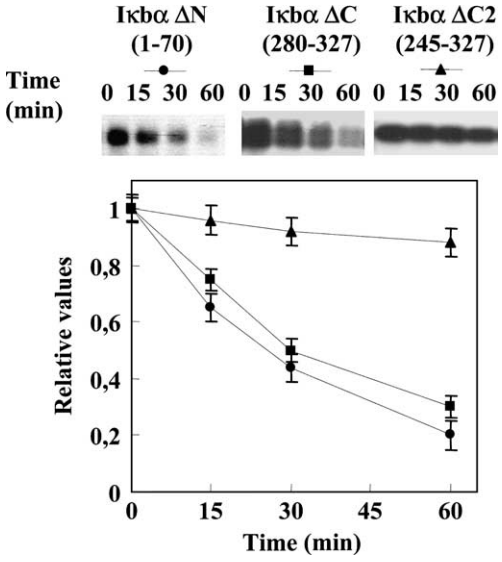

Fig. 3. Degradation of deletion constructs of IκBα by 20S proteasome. N- and C-terminal deletion constructs of IκBα were obtained by in vitro transcription and translation and subjected to degradation by 20S proteasome. Upper part of the figure shows a representative autoradiogram of the results obtained. Lower part of the figure shows the quantification of the degradation of the different constructs, results are referred to the amount of protein present at time 0 (identical values were observed in the absence of proteasome or in the presence of 10 μM lactacystin after incubation for 60 min, not shown), values represented are means ± S.D. from three different experiments.

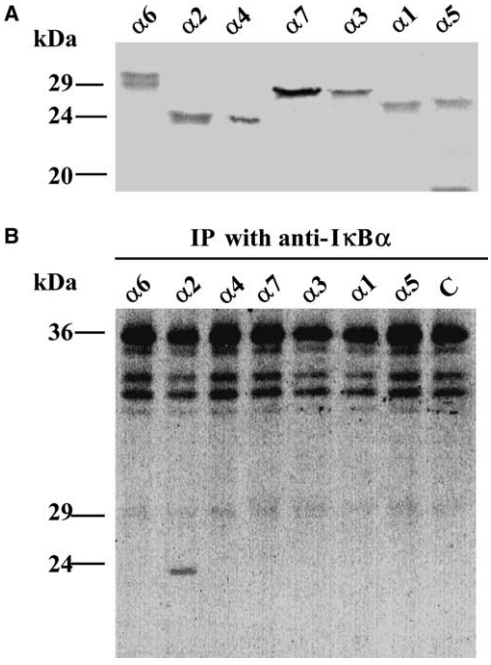

Fig. 4. Binding of IκBα to proteasomal alpha subunits. A, shows an autoradiogram of the in vitro transcribed and translated proteasomal alpha subunits used in the immunoprecipitation experiments. B, shows the autoradiogram of immunoprecipitation experiments with anti-IκBα antibodies of incubation mixtures that contained the different proteasomal alpha subunits (10 μl) as indicated and identical input of in vitro transcribed IκBα (5 μl), see Section 2 for full experimental details.

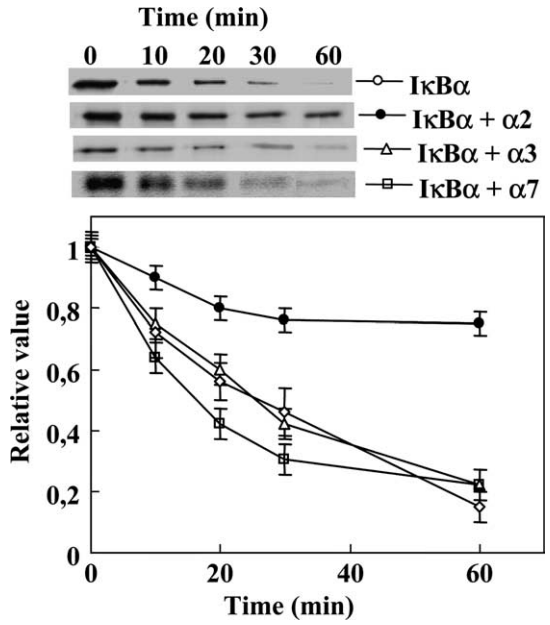

Fig. 5. Effect of different proteasomal alpha subunits on the degradation of IκBα by 20S proteasome. Upper part shows representative autogradiograms of time course of the degradation of IκBα by 20S proteasome in the absence or in the presence of recombinant α2, α3, and α7 proteasomal subunits. Lower part shows the quantification of the time-course of degradation, results are referred to the amount of protein present at time 0, values represented are mean ± S.D. from three different experiments.

proteasomal subunits and checked their effect on the degradation of IκBα by 20S proteasome. Fig. 5 clearly shows that α2 subunit, but not α3 or α7, is able to almost completely inhibit the degradation of IκBα by the 20S proteasome. Furthermore inhibition of IκBα by α2 subunit is dose-dependent with half-maximal inhibition at 1 μM, and no inhibition was found with up to 4 μM of protesomal α3 or α7 subunits (data not shown). These results indicate that proteasomal α2 (C3) subunit is implicated in the recognition of IκBα by 20S proteasome.

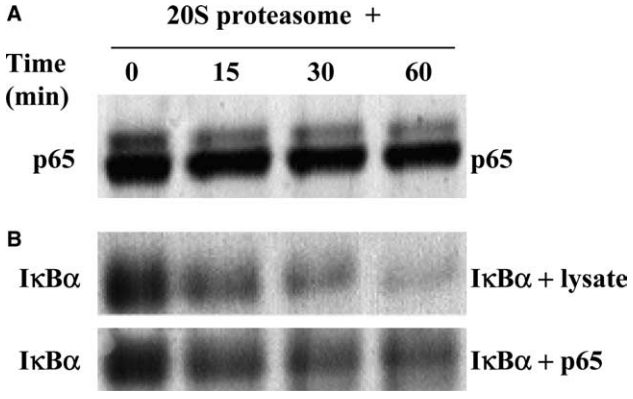

Fig. 6. Effect of p65 on the degradation of IκBα by 20S proteasome. A, shows a representative autoradiogram of a time-course of the incubation of p65 with 20S proteasome. B, shows a representative autoradiogram of the degradation of IκBα by 20S proteasome in the absence (an equal amount of mock lysate was added to have a similar protein load in these control reactions as in the next set of reactions with p65), or in the presence of rabbit reticulocyte lysate containing p65.

3.3. Effect of p65 on the degradation of IκBα by 20S proteasome

We have shown that IκBα can be directly degraded by 20S proteasome, but within the cells IκBα is associated with c-Rel family of transcription factors, mainly p65/RelA. As a consequence, it is interesting to study the degradation of p65 by the proteasome and its possible effect on the degradation of IκBα. Fig. 6A shows that p65 is not significantly degraded by incubation with 20S proteasome. When degradation of IκBα was studied in the presence of equal amounts of lysates containing or not containing in vitro transcribed/translated p65, we observed that the presence of p65 (Fig. 6B) greatly reduced the degradation of IκBα by the proteasome (70 ± 10% inhibition, from three different experiments). These results clearly indicate that association of IκBα with p65 clearly interfere with the direct degradation of IκBα by 20S proteasome.

4. Discussion

We have shown that IκBα, either recombinant or as product of in vitro transcription and translation, is degraded by the 20S proteasome. This degradation seems to proceed by binding of IκBα through its ankyrin repeats (ankyrin repeat VI is essential) to the α2(C3) subunit of the proteasome, as can be specifically inhibited by recombinant α2(C3) subunit, but not by recombinant α3 and α7 subunits. Trying to further delineate the region of α2(C3) responsible for binding to IκBα using C-terminal or N-terminal deletion constructs of α2(C3) have failed, likely indicating that the binding sites on the α2(C3) are not restricted to a single linear sequence of the polypeptide. This situation is similar to the degradation of p21 by 20S proteasome, where full length α7 inhibits the degradation of p21, but smaller constructs (with N or C-terminal deletions) were ineffective [35].

IκB protein family regulate NF-κB activation by sequestering NF-κB in the cytoplasm. IκBα is the key regulator of rapid signal-induced activation of NF-κB. The classical pathway is initiated with the activation of the IκBα kinases (IKK1 and IKK2) by pro-inflamatory citokines. The rapid phosphorylation of IκBα at serines 32 and 36 targets IκBα to polyubiquitylation by Ubc5/ SCF (beta-TrCP1) and then to degradation by the 26S proteasome, the degradation of IκBα exposed NFκB's nuclear localization signal and allowing NF-κB to translocate to the nucleus where activates transcription of many genes, including IκBα [6,26–28]. Apart of this signal dependent pathway of degradation of IκBα, several reports have analyzed the so-called signal-independent (basal) pathway of degradation [19,20,29,30]. From the studies by Krappmann et al. [19], it was concluded that basal turnover of IκBα does not require Ser 32 and 36 phosphorylation nor ubiquitylation of IκBα, as mutations of Ser 32 and 36 to Ala and of all Lys of IκBα to Arg, do not prevent its signal-independent degradation in transfected cells. Similarly, Pando and Verma [30] concluded that phosphorylation of Ser-32 and 36 is not required for the signal-independent turnover of IκBα. The in vitro data of IκBα degradation presented here perfectly agree with the above mentioned studies performed in cells and extend previous observation of IκBα degradation by 20S proteasome [20]. Furthermore, we report here that p65 is not a direct substrate of the 20S proteasome, but nevertheless protects IκBα from being degraded by the

proteasome, likely by binding of IκBα to p65 through its ankyrin repeats, prediction made on the basis of the X-ray structure of the complex [36,37]. These in vitro results are also in agreement with the reports of Krappmann et al. [19] and Pando and Verma [30], demonstrating that p65 prevents signal independent IκBα degradation [19]; and that free IκBα (mutC or S3236AmutC, unable to bind to NF-κB) is at least 5 times more rapidly degraded in the absence of external signals when compared with NF-κB-bound IκBα [30]. As a consequence the in vitro degradation data presented here recapitulate those obtained in studies of IκBα degradation under signal-independent (basal) conditions in intact cells. Previous and present works allows to conclude that signal independent (basal) degradation of IκBα can be directly mediated by 20S proteasome through specific interaction of IκBα with the α2(C3) proteasomal subunit and its rate of degradation is controlled by the association of IκBα with p65.

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