A mechanistic insight into a proteasome-independent constitutive inhibitor κBα (IκBα) degradation and nuclear factor κB (NF-κB) activation pathway in WEHI-231 B-cells

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Inducible activation of the transcription factor NF-κB (nuclear factor κB) is classically mediated by proteasomal degradation of its associated inhibitors, IκBα (inhibitory κBα) and IκBβ. However, certain B-lymphocytes maintain constitutively nuclear NF-κB activity (a p50–c-Rel heterodimer) which is resistant to inhibition by proteasome inhibitors. This activity in the WEHI-231 B-cell line is associated with continual and preferential degradation of IκBα, which is also unaffected by proteasome inhibitors. Pharmacological studies indicated that there was a correlation between inhibition of IκBα degradation and constitutive p50–c-Rel activity. Domain analysis of IκBα by deletion mutagenesis demonstrated that an N-terminal 36-amino-acid sequence of IκBα represented an instability determinant for constitutive degradation. Moreover, domain grafting studies indicated that this sequence was sufficient to cause IκBβ, but not chloramphenicol acetyltransferase, to be rapidly degraded in WEHI-231 B-cells. However, this sequence was insufficient to target IκBβ to the non-proteasome degradation pathway, suggesting that there was an additional *cis*-element(s) in IκBα that was required for complete targeting. Nevertheless, the NF-κB pool associated with IκBβ now became constitutively active by virtue of IκBβ instability in these cells. These findings further support the notion that IκB instability governs the maintenance of constitutive p50–c-Rel activity in certain B-cells via a unique degradation pathway.

Key words: B-cell, constitutive nuclear factor κB, inhibitor κBα (IκBα), inhibitor κBβ (IκBβ), nuclear factor κB (NF-κB), proteasome.

INTRODUCTION

Mammalian cells contain five members of the NF-κB (nuclear factor κB)/Rel family of proteins, p50, p65 (RelA), c-Rel, RelB and p52, which form diverse homo- or hetero-dimeric complexes [1]. NF-κB dimers operate as transcriptional regulators to affect either the inducible or constitutive expression of a wide array of genes involved in such cellular processes as inflammation, apoptosis and immune cell development and responsiveness [2]. Cells of the B-lymphocyte lineage, the context in which NF-κB was originally discovered, have long been noted to acquire constitutive NF-κB activity [3]. This activity can be comprised of a p50–c-Rel heterodimer [4–7], dimers containing the RelA subunit [8] or those containing p52 and RelB [9]. Based primarily on gene knock-out and transgenic mice studies, NF-κB dimers comprised of c-Rel or RelA on one hand, and p50 or p52 on the other, have been critical for proper development and activation of B-cells, in part by promoting immature B-cell survival [10]. Whereas IKKα [IκB (inhibitor κB) kinase α] and NIK (NF-κB-inducing kinase) have been directly linked to the control of constitutive p52 production in B-cells [11–13], the biochemical mechanisms that oversee the constitutive activity of complexes comprising c-Rel or RelA are not well understood.

Biochemical activity of NF-κB complexes comprising RelA or c-Rel is typically regulated by the IκB family of proteins, most notably IκBα and IκBβ [1]. A high affinity for dimeric RelA and c-Rel molecules enables these inhibitory proteins to associate with and thereby restrict the nuclear uptake of these NF-κB members. Because of this, nearly all known RelA- and c-Rel-inducing pathways proceed via the prior inactivation of IκBα and IκBβ proteins by the ubiquitin–proteasome pathway [1]. Thus a hallmark of the prototypical signal-inducible IκB-degradation and NF-κB-activation pathways is the sensitivity to a variety of cell permeant proteasome inhibitors. However, constitutive NF-κB activity seen in normal B-cells, differentiating B-cells *in vitro* or certain B-cell lines, such as WEHI-231, are highly refractory to inhibition by various proteasome inhibitors [14–16]. These observations suggested that a unique uncharacterized mechanism is involved in constitutive NF-κB activation in B-cells. Previous studies have also implicated changes in the composition of NF-κB dimers [9], an alteration in the phosphorylation status of IκBβ [17], degradation of IκBβ [18], an IκB-degradation-independent mechanism [19] or differences in the export activities of different NF-κB dimers [20] to mediate constitutive NF-κB activation in B-cells. Our previous studies have found that IκBα, but not IκBβ, is rapidly and constitutively degraded in WEHI-231 cells. Additionally, there was a correlation between IκBα degradation and constitutive NF-κB activation [14–16], suggesting that rapid IκBα turnover is critical for maintaining constitutive NF-κB activity. For example, constitutive IκBα degradation is resistant to several proteasome inhibitors, but sensitive to calcium chelators and CaM (calmodulin) inhibitors. Accordingly, constitutive

Abbreviations used: AAF-CMK, Ala-Ala-Phe-chloromethylketone; ALLnL, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal; ALLnM, *N*-acetyl-L-leucyl-L-leucyl-L-methioninal; BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl ester); CaM, calmodulin; CAT, chloramphenicol acetyltransferase; E-64d, (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; EMSA, electrophoretic mobility shift assay; IκB, inhibitor κB; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase; Tos-Lys-CH_2Cl, tosyl-lysylchloromethane; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane; Z-, benzyloxycarbonyl; Z-DEVD-FMK, Z-Asp-Glu-Val-Asp-fluoromethylketone; Z-VAD-FMK, Z-Val-Ala-DL-Asp-fluoromethylketone; Z-YVAD-FMK, Z-Tyr-Val-Ala-Asp-fluoromethylketone.

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NF-κB activation also displayed similar sensitivity profiles. Previous pharmacological studies also suggested that a calcium-dependent protease, calpain, might be playing a role in this unique IκBα-degradation pathway, because certain calpain inhibitors partially blocked constitutive IκBα degradation.

In the present study, we performed further evaluation of pharmacological agents and various IκBα mutants expressed in WEHI-231 B-cells. These studies revealed that this novel degradation pathway was unlikely to be mediated by calpain. Moreover, we found that an N-terminal 36-amino-acid sequence of IκBα could direct IκBβ to be rapidly degraded in WEHI-231 B-cells, but this sequence was insufficient for complete targeting to the non-proteasome-degradation pathway. Interestingly, the NF-κB pool associated with IκBβ became constitutively active by virtue of the instability conferred by the IκBα sequence. These findings indicate that there is more than one *cis*-element in IκBα required for efficient targeting to this unique non-proteasome pathway and that it is possible to alter the mechanism of constitutive NF-κB regulation by targeting a different IκB member for degradation. These observations further support the notion that degradation of IκB is essential for maintaining constitutive p50–c-Rel activity in WEHI-231 B-cells.

EXPERIMENTAL

Cell culture and chemicals

WEHI-231, W231-Bcl-X_L and W231-αN36-IκBβ9 cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 50 μM β-mercaptoethanol and antibiotics as described previously [15]. Media for W231-Bcl-X_L cells were supplemented with 0.5 mg/ml G418 (Mediatech) and those for W231-αN36-IκBβ9 cells received 0.5 mg/ml hygromycin B (Mediatech). Primary splenocytes were isolated from C57BL/6 mice as described previously [14]. Cycloheximide, NH_4Cl, bacterial LPS (lipopolysaccharide), Tos-Phe-CH_2Cl (tosylphenylalanylchloromethane, 'TPCK'), Tos-Lys-CH_2Cl (tosyl-lysylchloromethane, 'TLCK'), ALLnL (*N*-acetyl-L-leucyl-L-leucyl-L-norleucinal), ALLnM (*N*-acetyl-L-leucyl-L-leucyl-L-methioninal), Z-leucyl-leucyl-phenylalaninal (where Z is benzyloxycarbonyl), Z-leucyl-leucyl-norvalinal, Z-leucyl-leucyl-leucinal, EGTA, pyrrolidine dithiocarbamate, cyclosporin A, wortmannin and epoxomicin were purchased from Sigma–Aldrich. Z-glycyl-prolyl-phenylalanyl-leucinal and Z-glycyl-prolyl-phenylalanyl-phenylalaninal were generously provided by Dr Marian Orlowski (Department of Pharmacology, Mount Sinai School of Medicine, New York, NY, U.S.A.). The PR39 peptide was generously given by Dr Michael Simons (Angiogenesis Research Center, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, U.S.A.). Z-DEVD-FMK (Z-Asp-Glu-Val-Asp-fluoromethylketone) Z-VAD-FMK (Z-Val-Ala-DL-Asp-fluoromethylketone) and Z-YVAD-FMK (Z-Tyr-Val-Ala-Asp-fluoromethylketone) were purchased from Alexis Biochemicals. MG132 (Z-leucyl-leucylleucinal) was purchased from Peptide Institute, Inc. Calpeptin (Z-leucyl-norleucinal), E-64d [(2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester], PD150606, bisindoylmaleimide, KN62, KN93, BAPTA/AM [bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis-(acetoxymethyl ester)], W13 [*N*-(4-aminobutyl)-5-chloro-1-naphthalenesulphonamide], W12 [*N*-(4-aminobutyl)-1-naphthalenesulphonamide], W7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide], calmidazolium, FK-506, AAF-CMK (Ala-Ala-Phe-chloromethylketone) and *clasto*-lactacystin β-lactone were purchased from Calbiochem.

Mutagenesis and infections

N-terminal truncation mutants of murine IκBα were generated by PCR using appropriate primer sets. A 39-amino-acid C-terminal truncation mutant of murine IκBα has been described previously [21]. The αN36-CAT (chloramphenicol acetyltransferase) was also generated by two-step PCR and subcloned as described for αN66-CAT [22]. Substitution mutagenesis was performed by two-step PCR and all constructs were directly sequenced to verify integrity. The DNA encoding various forms of IκBα, as well as those encoding CAT derivatives, were all ligated into the pLHL-CA retroviral vector [21] and infection of WEHI-231 cells was as carried out as described previously [15].

Cell treatment, immunoprecipitation, Western blotting, EMSA (electrophoretic mobility shift assay) and apoptosis assays

Before all treatments, cells were allowed to incubate $\geqslant$ 4 h, once they had been aliquoted at (1.0–1.5) $\times$ 10^6 cells/ml, because we found that changing media led to weak, but proteasome-dependent, NF-κB activation, which subsided after approx. 2 h. For co-immunoprecipitation experiments approx. 1.5 $\times$ 10^6 cells, either treated or untreated, were lysed in hypotonic buffer A [23] supplemented with aprotonin and 1 mM PMSF. Following centrifugation, the pelleted nuclei were resuspended in buffer C [23], also supplemented with protease inhibitors. The resultant supernatant representing nuclear extract was diluted in TNEN buffer [10 mM Tris/HCl (pH 7.5)/150 mM NaCl/1mM EDTA/0.4 % Nonidet P-40] and split into two fractions for immunoprecipitation. Where samples were not immunoprecipitated, cells were lysed in total cell extract buffer [20 mM Hepes (pH 7.9)/350 mM NaCl/20 % glycerol/1 % Nonidet P-40/1 mM $MgCl_2$/0.5 mM EDTA/0.1 mM EGTA/0.5mM dithiothreitol] supplemented with protease inhibitors. Samples were separated by SDS/PAGE and Western blotted as described previously [16] with anti-IκBα (C21), anti-IκBβ (C20) or anti-(β-actin) (C-11) antibodies obtained from Santa Cruz Biotechnology. EMSA and supershifts were performed using an Igκ-κB oligonucleotide and antibodies obtained from Santa Cruz Biotechnology, as described previously [16]. All cycloheximide treatments were performed at 25 μg/ml for indicated times. Apoptosis assay was carried out by DNA laddering, as described previously [16].

Labelling *in vivo*

Pulse-chase experiments using WEHI-231 cells were performed as described previously [15] using antibodies against C-terminal IκBα (C21), N-terminal IκBα (5432) or C-terminal IκBβ (C20), and Protein A–Sepharose. IκB protein bands were visualized and quantified by PhosphorImager analysis using ImageQuant software (Molecular Dynamics).

RESULTS

Constitutive IκBα degradation in WEHI-231 cells does not require the C-terminal PEST sequence critical for calpain degradation *in vitro*

Based on our previous pharmacological findings, we thought that the calcium-dependent cysteine protease calpain might be involved in IκBα degradation in B-cells [15]. Indeed, Shen et al. [24] found that certain calpain inhibition could partially interfere with the degradation of IκBα and reduce constitutive NF-κB activity. Since we have found that the C-terminal 39 amino acids, including the PEST sequence, of IκBα are necessary for direct degradation by μ-calpain [22], we initially expressed an IκBα mutant lacking the C-terminal 39 amino acids in WEHI-231 cells

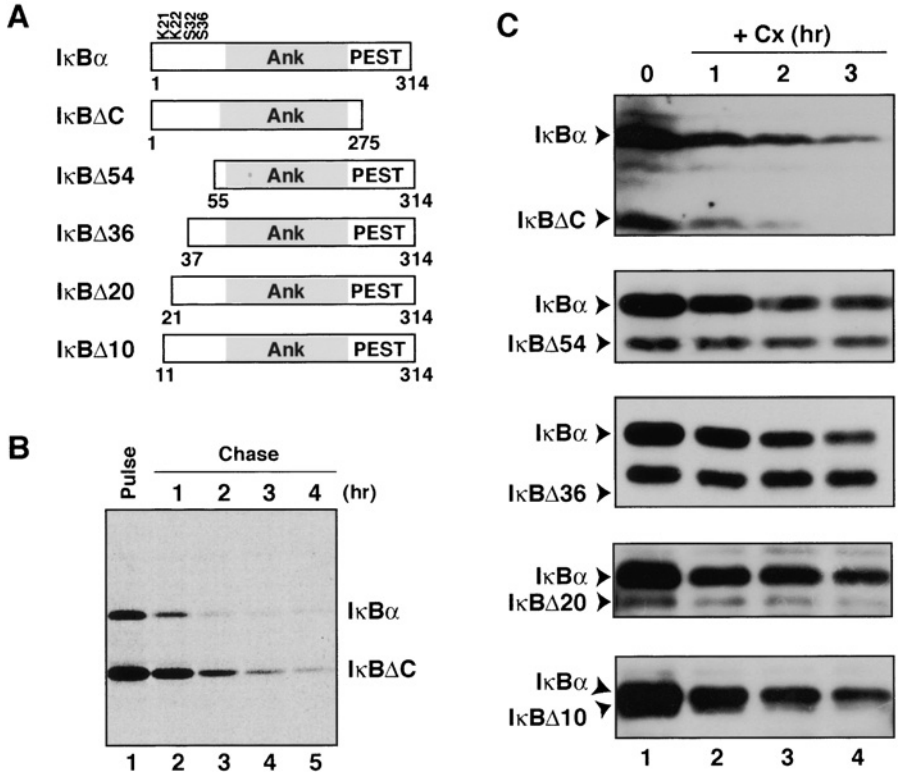

Figure 1 Sequences in the N-terminus, but not the C-terminal PEST, of IκBα are critical for its rapid turnover in B-cells

(**A**) Diagram of various IκBα mutants analysed for degradation in WEHI-231 cells. Representations of the wild-type and truncated proteins are given to the right of each panel. Ank represents the ankyrin repeats and PEST represents the PEST domain. (**B**) Pulse-chase analysis of IκBΔC (PEST deletion) mutant. The IκBΔC mutant protein was stably expressed in WEHI-231 cells and its half-life was measured by pulse-chase analysis using a pool of stable cells. IκBΔC was detected with antibody against the N-terminus of IκBα (5432). (**C**) IκBα proteins lacking the indicated region were stably expressed in WEHI-231 cells expressing the anti-apoptotic Bcl-X_L gene (W231.Bcl-X_L). Expression of the Bcl-X_L gene did not affect the IκBα-degradation pathway [22]. A pool of cells expressing each of the mutant proteins was treated with 25 μg/ml cycloheximide (Cx) for 0–3 h, and extracts separated by SDS/PAGE for Western blot analysis. IκBα proteins were detected with antibody against the C-terminus of IκBα (C21). Representative blots for each mutant are shown. Various exposure times of each confirmed that the endogenous IκBα protein turnover was similar in each case (results not shown).

(see Figure 1A for a diagram of the construct). The PEST deletion construct [22] was cloned into the pLHL-CA retroviral vector and it was then co-transfected into HEK293 cells along with the pCLeco helper construct. WEHI-231 cells were then added to the adherent HEK293 culture 24 h post-transfection and co-cultured for additional 24 h in the presence of polybrene to infect the suspension of WEHI-231 cells. The WEHI-231 cells were then removed from the culture and selected for pools of stable cells by the presence of hygromycin. The PEST-deletion mutant of IκBα was efficiently degraded in these stable cells, similar to the endogenous counterpart (Figure 1B) in a manner insensitive to proteasome inhibitors (results not shown). If calpain were directly involved in the constitutive degradation of IκBα, we would expect the PEST-deletion mutant to be stable in B-cells. Thus, the major proteolytic activity responsible for constitutive IκBα degradation appeared to be distinct from calpain in WEHI-231 B-cells (also see below).

N-terminal 36-amino-acid sequence of IκBα is necessary for constitutive degradation and can cause IκBβ to turnover rapidly

Similar to the C-terminal PEST deletion mutant, an IκBα mutant lacking the first 20 amino acids underwent rapid proteolysis in WEHI-231 cells (Figure 1C). However, a deletion of the first 36 or 54 amino acids of IκBα generated a stable protein. This indicated that the N-terminal domain of IκBα contained information

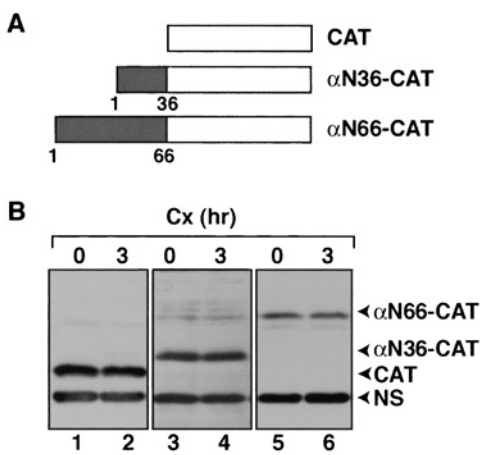

Figure 2 IκBα N-terminus fails to direct the rapid degradation of CAT in B-cells

(**A**) Schematic representation of chimeric proteins analysed. The shaded box represents sequences derived from IκBα. (**B**) The N-terminal 36 or 66 amino acids of IκBα were joined to CAT and these proteins were stably expressed in W231.Bcl-X_L cells. Protein amount was analysed by Western blotting with anti-CAT antibody with extracts from cells before or after the addition of 25 μg/ml cycloheximide (Cx) for 3 h. Left-hand panel, CAT; middle panel, αN36–CAT; right-hand panel αN66–CAT. NS refers to a non-specific band detected by the CAT antibody.

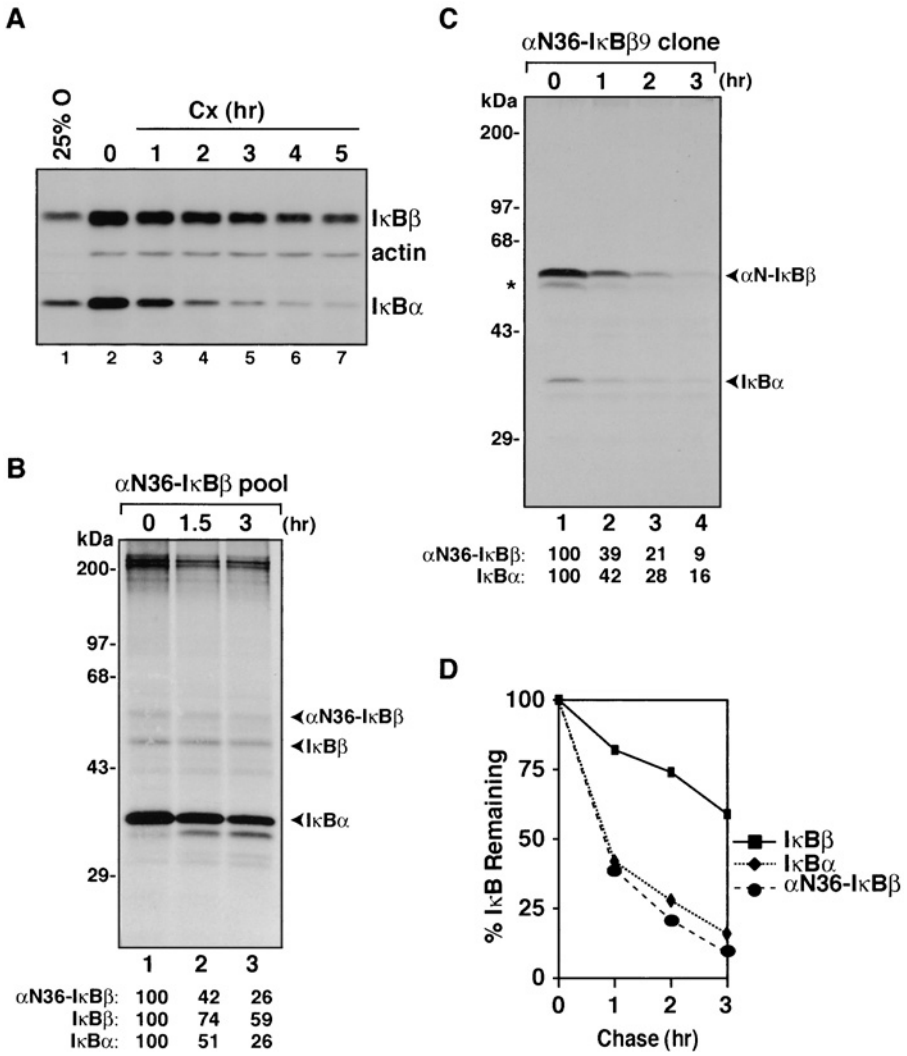

Figure 3 IκBα N-terminus is able to cause IκBβ to degrade rapidly in B-cells

(**A**) WEHI-231 cells were treated with cycloheximide (Cx) for the indicated times and analysed by Western blotting using antibodies specific to IκBα, IκBβ and β-actin. One-fourth of the untreated control cell extract (denoted as '0' in lane 2) was also loaded in lane 1 (denoted as '25 % 0') for comparison. Corresponding bands are indicated. (**B**) Turnover of αN36–IκBβ resembles that of IκBα. The turnover rates of IκBα, IκBβ and αN36–IκBβ were analysed by pulse-chase in a WEHI-231 pool of cells stably expressing the αN36–IκBβ protein. Immunoprecipitations of the labelled protein were done using the anti-IκBα antibody (C21) together with the anti-IκBβ antibody (C20), and specificities of the bands were confirmed with single antibody immunoprecipitations (results not shown). The relative amount of a specific protein remaining after the indicated chase period was determined by PhosphorImager analysis and is indicated below each lane. (**C**) αN36–IκBβ is rapidly degraded in a high-expressing clone. A clone (W231-αN36-IκBβ9) which contains a relatively large amount of αN36–IκBβ was isolated from the stably infected pool of WEHI-231 cells and analysed by pulse-chase as in (**B**), except that labelled proteins were immunoprecipitated with antibody against the N-terminus of IκBα (5432). A slightly faster migrating band (indicated by the asterisk) is likely to be derived from the chimeric protein. Numbers below the lanes indicate relative protein amount remaining after the indicated chase period. (**D**) Turnover rates of IκBα, IκBβ and αN36–IκBβ. Results from the pulse-chase experiment in (**C**), as well as from previous work [15], were plotted for comparison of degradation measurements.

necessary for its rapid degradation in these cells. To determine whether the IκBα N-terminus was sufficient to destabilize any protein in B-cells, we generated an αN36–CAT chimeric protein containing the N-terminal 36 amino acids of IκBα fused with the unrelated CAT protein (Figure 2A). This 36-amino-acid sequence of IκBα did not accelerate basal turnover of CAT (Figure 2B, lane 4). Even αN66–CAT, a chimeric CAT protein with the IκBα N-terminal 66 residues including a nuclear export sequence to force its expression in the cytoplasm [25], was also stable over three hours (Figure 2B, lane 6). This finding suggested that either the N-terminal IκBα sequence did not contain sufficient instability information or that this sequence needed to be in the context of an IκB protein to impart instability.

In WEHI-231 cells, constitutive degradation is selective only for IκBα among IκB family members and closely related IκB family member IκBβ is not turned over rapidly (Figure 3A, also see [15]). The N-terminal sequences of IκBα and IκBβ are nearly identical with respect to the IKK phosphorylation and ubiquitination sites, but are otherwise quite divergent, suggesting that there might be a *cis*-element(s) within the N-terminal 36-amino-acid sequence of IκBα that facilitated targeting to the non-proteasome pathway that was missing in IκBβ. To determine whether the N-terminal IκBα sequence was sufficient to destabilize IκBβ proteins, we generated an αN36–IκBβ chimeric protein containing amino acids 1–36 of IκBα fused to the N-terminus of full-length IκBβ. This chimera was stably expressed in WEHI-231 B-cells and its basal half-life was examined. To quantificatively measure the turnover rate, we first performed pulse-chase experiments with a polyclonal pool. Although its expression was low, it was clear from these experiments that the αN36–IκBβ

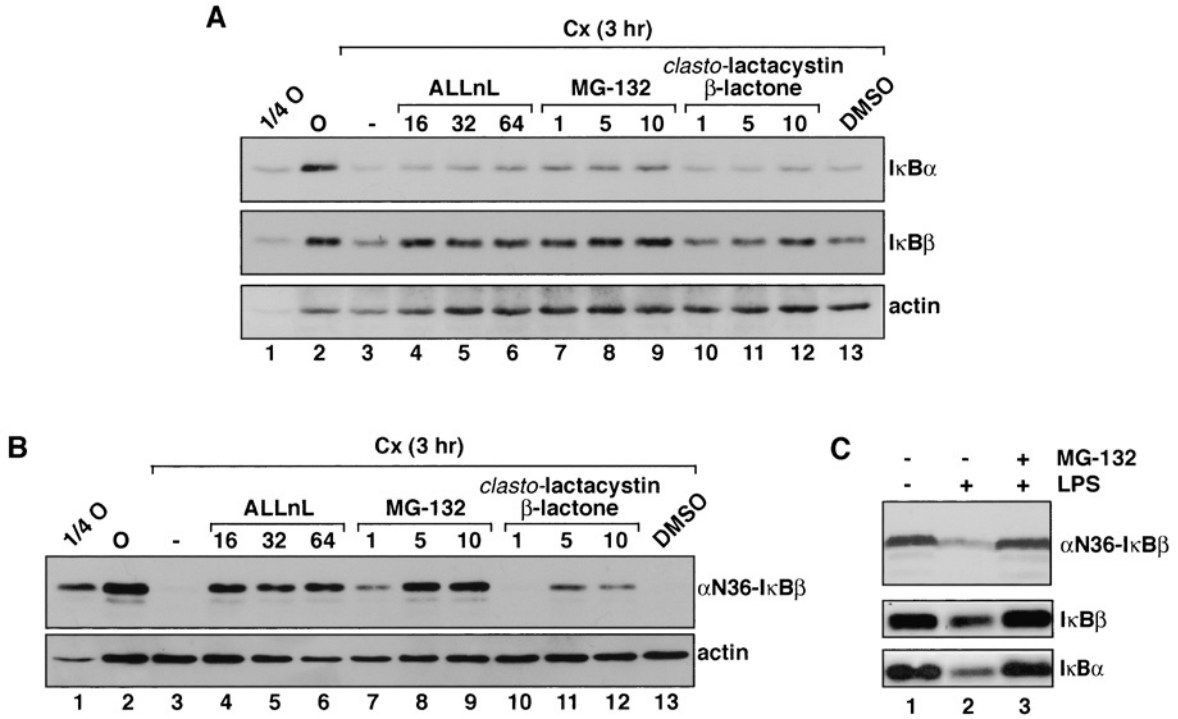

Figure 4 Rapid degradation of αN36–IκBβ is highly sensitive to low doses of proteasome inhibitors

(**A**) Turnover of IκBβ, but not IκBα, is extensively blocked by inhibitors of the proteasome. W231.Bcl-X_L cells were incubated with 25 μg/ml cycloheximide (Cx) for 3 h either alone (lane 3) or in the presence of ALLnL (amounts in μM, lanes 4–6), MG-132 (amounts in μM, lanes 7–9), *clasto*-lactacystin β-lactone (amounts in μM, lanes 10–12) or DMSO (lane 13). Total cell extracts were analysed by Western blotting for total IκBα, IκBβ or β-actin. One-fourth of the zero-time sample (lane 2) was included in lane 1 to aid in estimating the extent of degradation. Apparent low-level stabilization effects seen with IκBα proteins with certain proteasome inhibitors (lanes 4–12) are likely to be due to uneven loading, as judged by the loading control (β-actin). (**B**) Turnover of αN36–IκBβ is blocked by low doses of proteasome inhibitors. Experiment was performed as in (**A**), except that the W231-αN36-IκBβ9 clone was analysed after 3 h of treatment. αN36–IκBβ in the upper panel was detected using an antibidy against the IκBβ C-terminus (C20). (**C**) Degradation of αN36–IκBβ is induced by LPS, similar to the endogenous IκBα and IκBβ. W231.Bcl-X_L cells (for IκBα and IκBβ blots) and W231-αN-IκBβ9 clone (for αN36–IκBβ blot) were left untreated or treated with 10 μg/ml LPS in the absence (lane 2) or presence (lane 3) of 10 μM MG-132 for 30 min, and equivalent amounts of cell extracts prepared from these cells were analysed by Western blot analysis as described above.

half-life resembled that of IκBα, rather than that of IκBβ (Figure 3B). To further facilitate analysis of the αN36–IκBβ protein, we isolated a clone, W231-αN36-IκBβ9, which expressed a relatively high level of the transgene product. Importantly, the half-life of αN36–IκBβ in these cells was approx. 45 min (Figure 3C). This half-life was more rapid than that of IκBβ (>3 h) and it resembled the half-life of IκBα (approx. 40 min, see Figure 3D and [15]). Thus while amino acids 1–36 of IκBα failed to cause rapid degradation of the CAT protein, this sequence induced instability of IκBβ in WEHI-231 cells.

Rapid αN36–IκBβ degradation controls constitutive NF-κB activity in W231-αN36-IκBβ9 cells

We have shown previously that the degradation of IκBβ is relatively slow in WEHI-231 cells, and it is highly sensitive to low levels of proteasome inhibitors [15]. To determine whether the N-terminus of IκBα could target IκBβ to a non-proteasome pathway, we next evaluated the sensitivity of αN36–IκBβ to multiple proteasome inhibitors. Similar to the endogenous IκBβ protein, but unlike IκBα (Figure 4A), rapid degradation of αN36–IκBβ was readily blocked by low doses of these inhibitors (Figure 4B). Thus while the N-terminal sequence of IκBα was sufficient to cause instability in IκBβ, it was insufficient to cause complete targeting to the non-proteasomal pathway. Nevertheless, in the conventional pathway induced by LPS stimulation, the αN36–IκBβ fusion protein was similarly degraded to that seen with the endogenous IκBα and IκBβ (Figure 4C).

Since in W231-αN36-IκBβ9 cells the chimeric protein was the major IκB species expressed (Figure 3C), we next wanted to determine whether this degradation controlled constitutive NF-κB activity in these cells. We first examined whether constitutive NF-κB activities are similar to those found in primary splenocytes and the parental WEHI-231 cells. Figure 5(A) and 5(B) show that the constitutive NF-κB activity present in primary splenocytes displayed the similar lack of sensitivity to a potent proteasome inhibitor, ALLnL, and high sensitivity to calcium chelators, BAPTA/AM and EGTA, that is observed in WEHI-231 B-cells. Figure 5(C) shows that the major NF-κB complexes can be supershifted with anti-c-Rel and anti-p50 antibodies, but not by those specific to p65, RelB or p52, as seen in the primary and WEHI-231 B-cells [6,14,15]. Significantly, even though constitutive p50–c-Rel activity in parental cells is highly resistant to proteasome inhibitors (Figure 5D, lanes 3–6), W231-αN36-IκBβ9 cells became highly sensitized to proteasome inhibition (Figure 5D, lanes 9–12). This sensitization could be explained by the high sensitivity of degradation of the major IκB species, αN36-IκBβ, to these inhibitors in W231-αN36-IκBβ9 cells (Figure 4B). This conclusion was supported by the ALLnL sensitivity profiles of both the rapid αN36–IκBβ degradation and the constitutive NF-κB activity, which overlapped with each other (compare Figures 4B and 5D). Similar results were obtained with the proteasome inhibitors MG132 and *clasto*-lactacystin β-lactone (results not shown). Thus these results indicated that while the N-terminal IκBα sequence was insufficient for complete targeting of IκBβ to the non-proteasome degradation pathway, turnover of IκB was

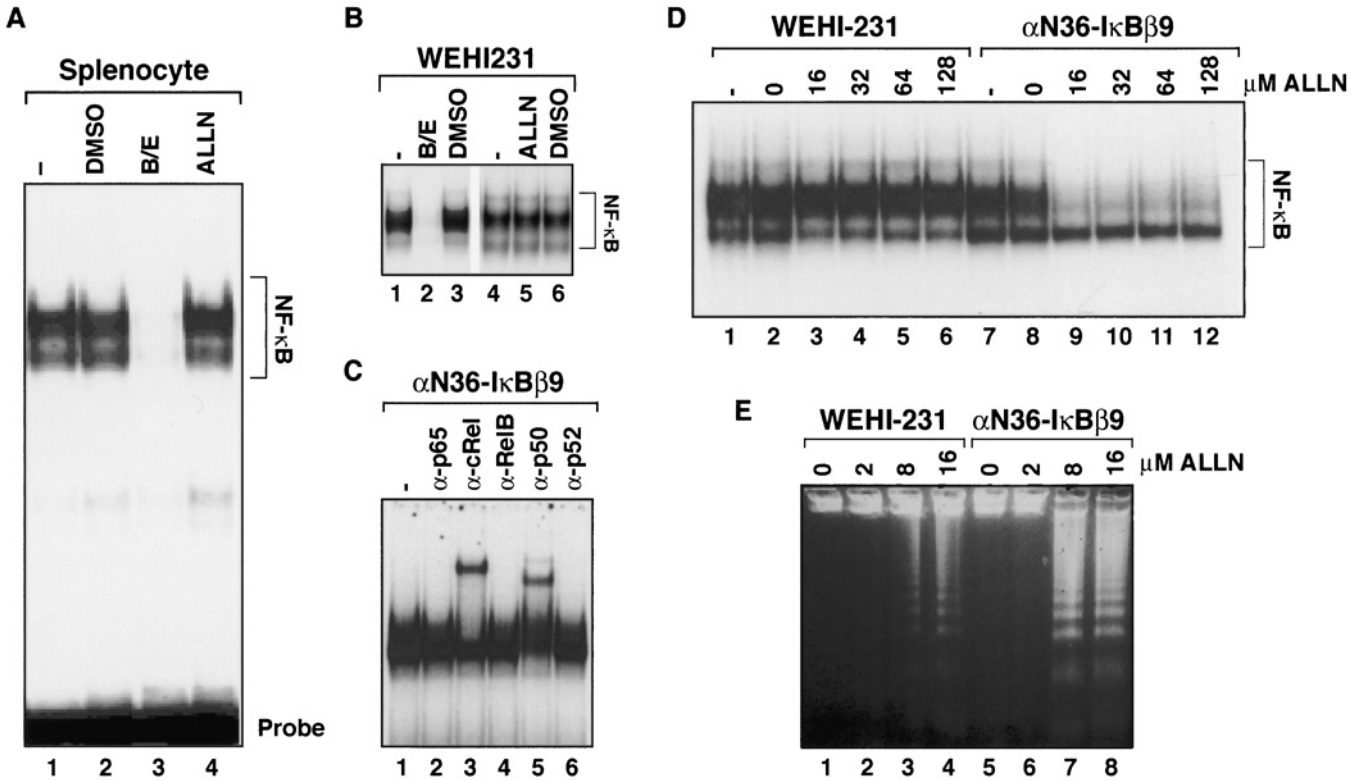

Figure 5 Sensitivity of constitutive NF-κB activity to proteasome inhibitors directly correlates with degradation of αN36–IκBβ

(**A**) Primary splenocytes freshly isolated from C57BL/6 mice were treated with DMSO, BAPTA/AM (30 μM) and EGTA (5 mM) (B/E), or ALLnL (ALLN; 50 μM) for 3 h and equivalent amounts of total extracts were then analysed by EMSA. The positions of different NF-κB complexes and free probe are indicated. (**B**) WEHI-231 cells were treated with the same inhibitors as above for 3 h and analysed as described above. (**C**) Supershift experiment was done using W231-αN36-IκBβ9 nuclear extracts with antibodies specific to each of the five NF-κB family members. (**D**) WEHI-231 or W231-αN36-IκBβ9 cells were incubated for 3 h in the presence of 16–128 μM ALLnL or with the carrier DMSO alone. Nuclear extract from each sample was examined for NF-κB DNA-binding activity by EMSA analysis. (**E**) Both WEHI-231 and W231-αN36-IκBβ9 cells were treated with indicated concentrations of the proteasome inhibitors for 5 h, and apoptosis-mediated DNA laddering was examined by agarose gel electrophoresis and revealed by ethidium-bromide staining.

causally involved in the maintenance of constitutive NF-κB activity in these B-cells. They also suggested the presence of yet an additional *cis*-element(s) within IκBα that was required for complete non-proteasomal targeting in this system. Finally, WEHI-231 cells that are partly resistant to proteasome inhibitors for induction of apoptosis, as measured by DNA laddering assay (Figure 5E, lanes 2–4), became highly sensitized to apoptosis induction by these inhibitors in W231-αN36-IκBβ9 cells (Figure 5E, lanes 7–8). This high sensitivity correlated with inhibition of constitutive NF-κB activity that provides a survival advantage to these cells [16,28].

Distinct chemical inhibitors affect constitutive IκBα degradation in WEHI-231 B-cells without interfering with the classical degradation pathway

A hallmark of the classical signal-inducible IκBα-degradation pathways is their sensitivity to cell-permeant proteasome inhibitors [2]. The results above showed that rapid constitutive IκBα degradation occurred despite the presence of high levels of several proteasome inhibitors in WEHI-231 B-cells, suggesting that the degradation of IκBα was mediated by a non-proteasomal pathway [14–16]. We have expanded these earlier studies with additional pharmacological agents. Consistent with our previous studies [14–16], all of the eleven proteasome inhibitors tested failed to efficiently prevent constitutive IκBα degradation in these B-cells (Table 1). These inhibitors included not only the typical β-catalytic subunit targeting inhibitors (e.g. ALLnL, ALLnM, MG132, lactacystin and *clasto*-lactacystin β-lactone), but also an inhibitor that targeted the non-catalytic α subunit of the proteasome core (PR39 [26]). This degradation was strongly inhibited by calcium chelators (BAPTA/AM and EGTA [15]), serine protease inhibitors (Tos-Phe-CH_2Cl and Tos-Lys-CH_2Cl [27]), CaM inhibitors (W13 and calmidazolium [16]), and an antioxidant (pyrrolidine dithiocarbamate [28]). However, it was resistant to a wide variety of chemical inhibitors, including a giant or tricorn protease inhibitor (AAF-CMK [29,30]), caspase inhibitors (Z-VAD-FMK, Z-DEVD-FMK and Z-YVAD-FMK), lysosomal inhibitors (NH_4Cl), calcineurin inhibitors (cyclosporin A and FK506), a CaM kinase inhibitor (KN62), a PKC inhibitor (bisindoylmaleimide) and a phosphoinositide 3-kinase inhibitor (wortmannin). While this degradation was also partially sensitive to a cysteine protease inhibitor (E64-d [15]), the calpain antagonist PD150606 had no inhibitory activity. This lack of inhibition by the calpain-selective inhibitor was consistent with our mutagenesis studies using the C-terminal PEST-deletion mutant (Figures 1B and 1C). These findings further supported the notion that a proteasome-independent, yet calcium/CaM-dependent mechanism is involved in degradation of IκBα protein in WEHI-231 B-cells.

DISCUSSION

While rapid progress has been made regarding our understanding of the mechanisms involved in signal-inducible activation of NF-κB, those governing constitutive NF-κB activity (a p50–c-Rel

Table 1 Pharmacological study of constitutive (non-classical) and LPS-inducible (classical) IκBα degradation and NF-κB activation in W231.Bcl-X_L B cells

W231.Bcl-X_L B cells were exposed to the indicated inhibitors for 30 min, then treated with cycloheximide for 3 h to block protein synthesis. The remaining proteins were analysed by Western blot analysis, as shown in Figure 1(C). Both 50 % and 25 % of the untreated samples were used as controls to evaluate the extent of degradation. Parallel cells were treated with the inhibitors (without cycloheximide) for 3 h and inhibition of p50/c-Rel activity was measured by EMSA. −, no inhibition; +/−, < 25 % inhibition; +, 25–50 % inhibition; ++, 50–100 % inhibition; ND, not determined; PDTC, pyrrolidine dithiocarbamate; Z-GPFF, Z-glycyl-prolyl-phenylalanyl-phenylalaninal; Z-GPFL, Z-glycyl-prolyl-phenylalanyl-leucinal; Z-LLF, Z-leucyl-leucyl-phenylalaninal; Z-LLL, Z-leucyl-leucyl-leucinal; Z-LLnV, Z-leucyl-leucyl-norvalinal.

Class	Compound (dose)	Observed inhibition	
		Non-classical	Classical
Proteasome inhibitors	ALLnL (1–100 μM)	−	++
	ALLnM (100 μM)	−	+
	Z-LLnV (50 μM)	−	++
	Z-LLL (5–50 μM)	−	++
	Lactacystin (1–100 μM)	−	++
	clasto-Lactacystin-lactone (1–25 μM)	−	++
	Epoxomicin (5–20 μM)	+/−	++
	Z-GPFL (10–100 μM)	−	+
	Z-GPFF (10–100 μM)	−	+
	Z-LLF (2 μg/ml)	−	+
	PR39 (2 μg/ml)	−	+
Serine protease inhibitors	Tos-Phe-CH_2Cl (2.5–25 μg/ml)	++	++
	Tos-Lys-CH_2Cl (2.5–25 μg/ml)	++	++
Lysosomal inhibitor	NH_4Cl (50 mM)	−	−
Giant/tricorn protease inhibitor	AAF-CMK (20 μM)	−	−
Caspase inhibitors	Z-VAD-FMK (5–50 μM)	−	−
	Z-DEVD-FMK (5–50 μM)	−	−
	Z-YVAD-FMK (5–50 μM)	−	−
Calpain/cysteine protease inhibitors	Calpeptin (10 μg/ml)	+/−	−
	E-64d (25 μg/ml)	+/−	−
	PD150600 (10–50 μM)	−	ND
Calcium chelators	BAPTA/AM (5–30 μM)	+/++	−
	EGTA (1–5 mM)	+/−	−
	BAPTA/AM + EGTA	++	−
Kinase inhibitors	Bisindoylmaleimide (20–200 nM)	+/−	ND
	Wortmannin (0.1–1 μM)	−	ND
	KN62 (10 μM)	−	ND
CaM antagonists	W13 (0.25–25 μM)	++	−
	W12 (5–200 μM)	+	−
	W7 (5–40 μM)	+/−	ND
	Calmidazolium (1–10 μM)	+	ND
Calcineurin inhibitors	Cyclosporin A (0.01–10 μg/ml)	−	ND
	FK506 (1–50 μM)	−	ND
Antioxidant	PDTC (100 μM)	++	++

heterodimer) in B-cells, the context in which it was first discovered, remain obscure. Mechanisms that control the activity of other forms of constitutive NF-κB complexes seen in B-cells, such as those containing p52 and RelB, have been described [9]. Previous studies by different laboratories provided further insights for p100 processing via an NIK–IKKα signalling pathway as the critical determinant in regulating p52–RelB activity [11,13,31,32]. However, NF-κB complexes composed of c-Rel and RelA can also be found in B-cells, but the biochemical mechanisms involved in their activation and maintenance in B-cell development are not well established. Activity of c-Rel and RelA are also critical to B-cell development, because B-cells do not properly develop beyond the immature B-cell stage when these NF-κB members are both deleted [33] or their activity attenuated by the expression of an IκBα mutant protein [34].

While the hallmark of the signal-inducible NF-κB activation pathways is their sensitivity to a variety of proteasome inhibitors, constitutive p50–c-Rel activity seen in many murine B-cell lines [15], primary splenic B-cells and conditionally differentiating B-cells *in vitro* [14] is highly refractory to these inhibitors. Coincidentally, IκBα is rapidly degraded in B-cells in a manner that is insensitive to high levels of proteasome inhibitors [14]. Our present studies extend these findings and show that IκBα can be rapidly degraded in WEHI-231 B-cells under conditions in which the signal-inducible pathway is completely prevented by proteasome inhibitors. We probed into the role of both N- and C-terminal domains of IκBα in this proteasome-inhibitor-resistant degradation in WEHI-231 B-cells. This mutational analysis (to identify critical *cis*-elements of IκBα) identified the N-terminal 20–36 amino acid sequence to be necessary for non-proteasomal degradation. We found that the N-terminal region of IκBα is sufficient to allow IκBβ, but not the CAT protein, to undergo a much more rapid degradation than endogenous IκBβ in WEHI-231 B-cells. This indicated that the N-terminal sequence of IκBα might only function in the context of IκB protein structure. This may be partly due to the necessity of IκB to be associated with NF-κB proteins, since free IκBα protein was found to be rapidly degraded via a ubiquitin-independent, but proteasome-dependent, pathway [35]. Interestingly, the IKK phosphorylation sites and ubiquitin conjugation sites are conserved between these two proteins, yet the addition of the IκBα N-terminus to IκBβ resulted in accelerated degradation of αN36–IκBβ fusion protein. Thus there might be a critical determinant within the IκBα N-terminus, other than the phosphorylation and ubiquitylation sites, which is missing in IκBβ.

Unlike IκBα, and like IκBβ, the rapid degradation of αN36–IκBβ was efficiently prevented by as little as 8–16 μM ALLnL. Most strikingly, when αN36–IκBβ is the major IκB protein expressed in these cells, constitutive NF-κB activity, which is normally refractory to even 100 μM ALLnL, is now extremely sensitized to this proteasome inhibitor at low doses (8–16 μM). Sensitivity of αN36–IκBβ degradation to various proteasome inhibitors (ALLnL, MG132 and *clasto*-lactacystin β-lactone) displayed direct overlap with that of constitutive NF-κB activity to these inhibitors in this cell system. Thus while the N-terminus of IκBα was insufficient to completely target IκBβ to the non-proteasome pathway, we were able to use the N-terminal IκBα instability determinant to (i) confer instability to IκBβ and (ii) cause constitutive NF-κB activation that is now dependent on IκBβ turnover in B-cells. These findings provide further evidence for a causal relationship between rapid IκB degradation and the maintenance of constitutive NF-κB activation in these B-cells.

Our previous studies [15] and the study by Shen et al. [24] suggested that the calcium-dependent protease calpain might be the proteolytic system that oversees IκBα degradation in B-cells. The requirement of calcium for its degradation and a modest inhibition by calpain inhibitors (ALLnL, ALLnM and calpeptin) and a cysteine protease inhibitor (E64-d) supported the potential involvement of calpain in IκBα degradation. Similar to our previous findings, Shen et al. [24] also found that both E-64d and calpeptin partially inhibited IκBα degradation and NF-κB activation in B-cells. Our independent approach to determine the involvement of calpain in IκBα proteolysis identified the C-terminal 39-amino-acid sequence that contains the PEST sequence to be critical for direct degradation by calpain *in vitro* [22]. The IκBα mutant protein missing this critical domain was degraded in these B-cells as efficiently as the wild-type counterpart in a manner still resistant to proteasome inhibitors, yet sensitive to calcium chelators (S. D. Shumway, unpublished work). Consistent with this lack of support for calpain involvement, we also did not see any inhibitory effect with yet another calpain inhibitor (PD150606), whose inhibitory activity targets the

calcium-binding domain of calpain [36], not the catalytic core like the aforementioned inhibitors. It is suggested that targeting the calcium-binding domain may provide more selectivity for calpain inhibition, since other calpain inhibitors could also target different proteolytic systems [36]. Both pharmacological and structure–function analyses of the IκBα protein suggested that the major proteolytic system that controls IκBα degradation in B-cells is unlikely to be calpain. The partial effects seen with the calpain inhibitors may be due to partial inhibition of the unknown proteolytic system involved in IκBα degradation. Our additional pharmacological studies using proteasome inhibitors targeting both the catalytic β-subunits and a non-catalytic α-subunit of the 20S core further supported the notion that the protease involved in IκBα degradation in WEHI-231 cells is distinct from proteasomes. We also tested an additional inhibitor that targets a recently identified proteolytic system, the giant [30], or tricorn [29], protease. This latter proteolytic activity was of particular interest, since growth of EL-4 lymphoma cells in the presence of proteasome inhibitors yielded cells that now degrade proteasome substrates by this system [29]. Thus the tricorn-protease system can complement the proteasome in certain mammalian cells. We wondered whether IκBα was degraded by this alternative degradation system in B-cells. However, we failed to demonstrate any effects using the inhibitor AAF-CMK. Similarly, the lack of effects with NH_4Cl indicates that lysosomal degradation is also unlikely to be the principal pathway. Thus the nature of the protease that oversees IκBα degradation in WEHI231 B-cells and primary B-cells remains to be elucidated. Further *cis*-elements in IκBα that are required for targeting to this proteolytic pathway may help to reveal the protease involved.

We thank Bradley Seufzer for technical support and the Miyamoto laboratory members for helpful discussions. This work was supported in part by funding from a National Institutes of Health (NIH) training grant (T32GM07215) to S. D. S. and NIH R01-CA77474, NIH R01-CA81065 and the Shaw Scientist Award from the Milwaukee Foundation to S. M.

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Received 22 November 2003/3 February 2004; accepted 6 February 2004
Published as BJ Immediate Publication 6 February 2004, DOI 10.1042/BJ20031796