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Phosphorylation by the Protein Kinase CK2 Promotes Calpain-Mediated Degradation of IκBα

Jian Shen,* Padmalatha Channavajhala, † David C. Seldin, † and Gail E. Sonenshein‡

Rapid IκBα turnover has been implicated in the high basal NF-κB activity in WEHI 231 B immature IgM⁺ B cells. Here we show that treatment of WEHI 231 cells with apigenin, a selective inhibitor of the protein kinase CK2, decreased the rate of IκBα turnover and nuclear levels of NF-κB. Turnover of IκBα in these cells is mediated in part by the protease calpain. Since both CK2 and calpain target the proline-glutamic acid-serine-threonine (PEST) domain, we investigated the role of CK2 in the degradation of IκBα by calpain using an in vitro phosphorylation/degradation assay. CK2 phosphorylation enhanced μ-calpain-mediated degradation of wild-type IκBα, but not of mutant 3CIκBα, with S283A, T291A, and T299A mutations in phosphorylation sites within the PEST domain. Roles for CK2 and calpain in IκBα turnover were similarly shown in CH31 immature and CH12 mature IgM⁺ B cells, but not in A20 and M12 IgG⁺ B cells. These findings demonstrate for the first time that CK2 phosphorylation of serine/threonine residues in the PEST domain promotes calpain-mediated degradation of IκBα and thereby increases basal NF-κB levels in IgM⁺ B cells. The Journal of Immunology, 2001, 167: 4919–4925.

Nucelar factor-κB is a family of dimeric transcription factors that have been found to play important roles in the development and activation of B lymphocytes (1, 2). NF-κB is constitutively expressed in the nucleus of mature and late immature B cells. In most non-B cells, NF-κB factors are normally sequestered in the cytoplasm via interaction with specific inhibitory proteins, termed IκBs. IκBs are a family of related proteins that include IκBα, IκBβ, IκBε, IκBγ/p105, and IκBδ/p100 (3). Activation of NF-κB is dependent on degradation of IκB proteins. Although the mechanisms controlling constitutive NF-κB expression in B cells are not clear, the rapid rate of degradation of IκBα was hypothesized to be a contributing factor in immature B lymphoma cells such as WEHI 231 (4). Interestingly, Doerre and Corley (5) showed more rapid decay of IκBα protein in IgM⁺ B cell lines (such as WEHI 231, CH31, and CH12 cells) vs IgG⁺ B cells (e.g., A20 and M12 cells), although the mechanism for these differences was not elucidated.

The proteasome pathway of IκBα proteolysis has been implicated in NF-κB activation via extracellular stimuli, such as TNF, LPS, and growth factors. Stimulation induces phosphorylation of IκBα on serines 32 and 36 by the IκB kinase complex (IKK) (6). This phosphorylation serves as a signal for subsequent ubiquitination, which promotes IκBα degradation by the 26S proteasome (7). This pathway is not universal, however, and proteasome-mediated degradation of IκBα has been ruled out in several recent cases (8–10). Turnover of IκBα mutant at the Ser12/36 was found to occur with the normal kinetics following induction of oxidative stress upon treatment with H2O2 (8). Furthermore, Miyamoto and co-workers (9) found that inhibitors of calpain, but not of the proteasome, ablated basal IκBα turnover in WEHI 231 cells.

Additional kinases have been implicated in the regulation of IκBα activity or stability. Protein kinase CK2 (previously known as casein kinase II) phosphorylates IκBα on serines and threonines in the proline-glutamic acid-serine-threonine (PEST) sequence domain, which affects the intrinsic stability of this inhibitory protein (11–14). Phosphorylation of Ser32, Thr286, Ser293, and Thr299 has been reported, although some controversy exists as to the nature of the important sites. Interestingly, the PEST domain of IκBα was also shown to be the target for degradation by μ-calpain (15). This led us to hypothesize that CK2 may play a role in IκBα degradation through the μ-calpain proteolytic pathway. Consistent with this hypothesis, here we report that treatment of WEHI 231, CH31, and CH12 IgM⁺ B cells with inhibitors of either CK2 (apigenin) or the calcium-calpain proteolytic pathway decreased the rate of IκBα decay and thereby NF-κB levels. Furthermore, in vitro phosphorylation of IκBα by CK2 enhanced its rate of degradation by μ-calpain. Thus, these studies define an important new step in the regulatory pathway controlling basal expression of NF-κB in IgM⁺ B cells.

Materials and Methods

Cell culture and treatment conditions

WeHI 231, CH31, and CH12 cells were maintained in DMEM as described previously for WEHI 231 (16). A20 and M12 cells were maintained in RPMI with 10% FBS, 50 μM β-mercaptoethanol, and 100 μg/ml streptomycin. Where indicated, apigenin (Sigma, St. Louis, MO), 1,2-BAPTA-AM (Novabiochem, La Jolla, CA), calpeptin (Calbiochem, La Jolla, CA), E-64d (Peptide International, Louisville, KY) dissolved in DMSO, or a similar dilution of DMSO as a control was added. For half-life studies, 15 μg/ml cycloheximide was added, and whole cell extracts (WCEs) were prepared in lysis buffer (20 mM Tris (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM p-nitrophenyl phosphate, 300 μM NaVO₄, 1 mM benzamidine, 2 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM DTT, and 0.25% Nonidet-P40) (17).

Immunoblot analysis of IκBα proteins

Samples of extracts (30 μg) were subjected to immunoblot analysis as previously described (18). The Abs used were IκBα (C-21), IκBβ (C-20), and thereby increases basal NF-κB levels in IgM⁺ B cells. The Journal of Immunology, 2001, 167: 4919–4925.
and p27 (C-19; Santa Cruz Biotechnology, Santa Cruz, CA). An mAb specific for the β-actin (AC-15) was purchased from Sigma. Resulting autoradiograms of decay were quantified by densitometry, and linear regression analysis was used to calculate the $t_{1/2}$. Shown are representative data from a minimum of two experiments.

**EMSA**

Nuclear extracts were prepared and subjected to gel electrophoresis as previously described (19). The sequence of the NF-κB-containing oligonucleotide from the c-my c gene is 5’-GATCCAGTGCGGTTTCCGACCC-3’, where the underlined region indicates the core binding element (20). The octamer-1 (Oct-1) oligonucleotide has the sequence 5’-TGGCGATGCAATCAGTAA-3’.

**Transient transfection analysis**

Exponentially growing WEHI 231 cells were electroporated with 30 μg of an NF-κB element-driven luciferase construct (21), provided by Georges Rawadi (Hoescht-Marion-Roussel, Romainville, France) and 5 μg SV40 β-galactosidase (β-gal) reporter construct. After electroporation, cells were incubated at 37°C for 8 h in the absence or the presence of 20 or 40 μM apigenin or 50 μg/ml of the calpain inhibitor E-64d. Cells were harvested, and luciferase activity was measured and normalized with β-gal activity.

**CK2 kinase assay**

For evaluation of IκBα phosphorylation directed by CK2, WCEs were prepared, and samples (10 μg) were adjusted to a 5-μl final volume with the CK2 kinase buffer (100 mM Tris (pH 8.0), 100 mM NaCl, 50 mM KCl 20 mM MgCl₂, and 100 mM Na₂VO₃). Following addition of 15 μl incubation buffer, including CK2 kinase buffer, 50 μCi [γ-32P]GTP, and 200 ng wild-type (wt) IκBα-GST or 3CIκBα-GST, with three point mutations at S283A, T291A, and T299A (20), provided by J. Hiscott (McGill University, Institut Lady Davis de Recherches Médicales, Montréal, Canada) protein, reactions were incubated at 30°C for 30 min. These fusion proteins were prepared as we have previously described (22). Where indicated, the CK2-specific peptide substrate RRREEETEEE or apigenin was added to the kinase reaction. Samples were subjected to gel electrophoresis and autoradiography. To assess equal loading of protein extracts or GST fusion proteins, the gel was rehydrated and stained with Coomassie blue. Alternatively, 1 mM of the CK2-specific peptide RRREEETEEE (Genosys Biotechnologies, The Woodlands, TX) was used as substrate, as described above, except that 5 μg WCEs were used, and the reactions were stopped by adding 25 μl 100 mM ATP in 4 N HCl. Samples were spotted onto a P81 Whatman filter (Clifton, NJ) and washed four times in 150 mM H₃PO₄, and incorporated radioactivity was measured by scintillation counting.

**Calpain treatment**

For CK2 phosphorylation, wtIκBα-GST or 3CIκBα-GST protein (50–100 ng) was adjusted to either 50 μCi [γ-32P]GTP or 1 mM ATP in 10 μl CK2 kinase buffer and treated with either 10 or 20 U recombinant CK2 (New England Biolabs, Beverly, MA), respectively, at 30°C for 15–30 min. After the kinase incubation, 10 μl of 2× calpain incubation buffer containing 1.5 mM DTT, 1.5 mM CaCl₂, and the indicated concentration of calpain I from human erythrocytes (Calbiochem) was added, and the mixture was incubated at 30°C for 15 min as described previously (15). The reaction was terminated by addition of an equal volume of 2× loading buffer and boiling for 5 min, and the proteins were subjected to autoradiography or immunoblot analysis, as appropriate.

**Results**

**Apigenin inhibits CK2 activity in WEHI 231 cells**

CK2 phosphorylates IκBα in the PEST domain, and mutation of S283A, T291A, and T299A was previously shown to reduce phosphoryl group transfer by CK2 in vitro (20). Thus, a CK2 kinase assay was developed using WEHI 231 WCEs and either wtIκBα-GST or mutant 3CIκBα-GST, with point mutations in critical serine and threonine residues, as substrate. Since either ATP or GTP can be used as a phosphate donor by CK2, while the IKKs can only use ATP, assays were performed in the presence of [γ-32P]GTP. Phosphorylation of the wt was substantially higher than that of the mutant IκBα fusion protein, consistent with CK2 kinase activity (Fig. 1A). The 3CIκBα-GST was phosphorylated in an in vitro IKK kinase assay to the same extent as wtIκBα-GST (data not shown). Addition of the CK2-specific peptide substrate RRREEETEEE or the selective inhibitor apigenin (23, 24) reduced this activity in a dose-dependent fashion (Fig. 1A). Equal IκBα and protein loading was confirmed by Coomassie staining. These findings verify that this assay monitors CK2 activity using IκBα as substrate.

To measure the effects of CK2 activity on NF-κB levels in WEHI 231 cells, we used the CK2 inhibitor apigenin. We first confirmed the effects of apigenin on CK2 activity in WEHI 231 cells. At a low dose of 20 μM, apigenin has displayed selective inhibition of CK2 in different cell lines (24), so this concentration was chosen initially. WEHI 231 cells were grown in the presence of 20 μM apigenin or an equivalent volume of carrier DMSO as a control. After either 3 or 8 h WCEs were isolated and subjected to an in vitro CK2 kinase assay using wtIκBα-GST protein as the substrate (Fig. 1B). Compared with DMSO-treated controls, a significant down-regulation of CK2 activity was measured by 3 h, and no additional decrease in activity was noted at the 8 h point. Thus, apigenin can effectively inhibit CK2 activity in WEHI 231 cells by 3 h of treatment.

We then performed a dose-response curve of the effects of apigenin on CK2 activity using either wtIκBα-GST or the consensus CK2 peptide as substrate. WEHI 231 cells were treated with 0, 10, 20, 30, or 40 μM apigenin for 3 h, and WCEs were prepared. Using these extracts in kinase assays, a dose-dependent decrease in activity was seen using IκBα-GST as a substrate (Fig. 1C). Equal IκBα and protein loading was confirmed by Coomassie staining. Similarly, apigenin was found to cause a dose-dependent decrease in CK2 activity when the CK2-specific peptide substrate RRREEETEEE was used (Fig. 1D). Thus, apigenin inhibits CK2 activity in WEHI 231 cells in a dose-dependent fashion.

**Apigenin selectively decreases IκBα stability and basal levels and activity of NF-κB**

To assess the effects of the inhibition of CK2 on the rate of decay of IκBα and IκBβ, WEHI 231 cells were treated with 20 μM apigenin or carrier DMSO for 4 h before addition of the protein synthesis inhibitor cycloheximide. Cytoplasmic proteins were isolated after 0, 30, 60, 120, and 180 min and subjected to immunoblot analysis (Fig. 2A), and the results of this and a duplicate experiment were quantified by densitometry (Fig. 2B). A slower rate of decay of IκBα protein was noted in the apigenin-treated cells ($t_{1/2}$ 101.4 ± 12 min) compared with DMSO-treated control ($t_{1/2}$ 50.4 ± 4.8 min) cells. In contrast, the stability of IκBβ was not altered in the same samples ($t_{1/2}$ 108.4 vs 100.7 min in control vs apigenin-treated cells, respectively; Fig. 2, A and B). To determine whether the increase in half-life of IκBα indeed correlated with a drop in NF-κB binding, WEHI 231 cells were treated with 20 μM apigenin or carrier DMSO for 8 h, and nuclear extracts were subjected to EMSA (Fig. 2C). NF-κB, but not control Oct-1, binding activity was down-regulated in nuclear extracts from cells treated with apigenin compared with control DMSO-treated cells. Previous Ab supershift analysis indicated that band 1 contains p50 homodimers, while band 2 consists predominantly of p50/c-Rel with lesser amounts of p50/RelA complexes (16). The down-regulation of NF-κB activity was also confirmed by lower luciferase activity in an NF-κB reporter assay in 3-h apigenin-treated WEHI 231 cells compared with untreated control cells (Fig. 2D). Taken together, the above observations suggest that apigenin can inhibit CK2 phosphorylation of IκBα, prolong IκBα half-life, and selectively down-regulate NF-κB levels and activity in WEHI 231 cells.
Recently, basal degradation of IκBα protein in immature B cells has been reported to be mediated via calpain (9, 10). To confirm this observation WEHI 231 cells were incubated in the presence of either 40 μg/ml calpeptin or 50 μg/ml E-64d, two specific inhibitors of calpain, or carrier DMSO as a control. After 30 min, cycloheximide was added, and cytoplasmic extracts were isolated after 2.5 h. Addition of either calpain inhibitor significantly decreased the extent of IκBα decay (Fig. 3A). Densitometry of this and a duplicate experiment indicated that the normal decay, which leaves 36.0 ± 5.7% of the IκBα remaining after 2.5 h, is ablated upon treatment with either calpeptin or E64d (88.9 ± 5.1 and 74.5 ± 10.7%, respectively). Equal loading of IκBα was confirmed by analysis of β-actin levels on the blot. Furthermore, addition of intracellular and extracellular calcium chelators (30 μM BAPTA-AM plus 5 mM EGTA (B/E)), which inhibits calpain activity (10), completely blocked IκBα degradation (106.1 ± 8.5%; Fig. 3A). To verify that these inhibitors were not working through the proteasome pathway, we examined the decay of the p27Kip1 protein, which is mediated via the proteasome (25). Normal degradation of this protein was seen in the presence of the calpeptin, E-64d, or calcium chelators. To assess NF-κB levels, WEHI 231 cells were incubated in the absence or the presence of E-64d (Fig. 3B). The level of NF-κB/Rel binding was reduced in a manner consistent with the increased stability of the IκBα protein. For the upper, transcriptionally active NF-κB complexes, approximately 60% remained following E64d treatment compared with the DMSO control. As expected, addition of the calcium chelators had a more substantial effect on NF-κB binding, consistent with their effects on IκBα half-life. Similarly, NF-κB reporter activity was also decreased in cells treated with 50 μg/ml E-64d for 8 h after transfection (Fig. 3C). Taken together, these results indicate calpain plays an important role in basal degradation of IκBα and thereby NF-κB levels in WEHI 231 cells.

**CK2 promotes degradation of IκBα by calpain**

Since CK2 phosphorylates IκBα in the C-terminal PEST domain, which is the target of calpain protease, we asked whether phosphorylation of IκBα by CK2 affects its rate of degradation by calpain. We first sought to determine the appropriate dose of μ-calpain needed. The wtIκBα-GST was phosphorylated using [γ-32P]GTP and recombinant CK2, and then the mixture was incubated for 15 min with 30, 90, or 180 nM μ-calpain in the presence or the absence of 750 μM calcium. Degradation of the radiolabeled protein was assessed by gel electrophoresis and autoradiography (Fig. 4A). In the presence of calcium, treatment with 30 nM μ-calpain resulted in a substantial decrease in radiolabeled wtIκBα-GST, and with 90 nM μ-calpain little remaining IκBα was detected. As expected, degradation by μ-calpain required calcium, and, hence, in the absence of this divalent cation, no degradation was detected even with the higher dose of 180 nM μ-calpain. Thus, phosphorylated IκBα-GST protein is efficiently degraded by calpain in vitro. Doses of 60 and 90 nM calpain were selected for the analysis.
Immunoblot analysis. WEHI 231 cells were incubated with 20 nM apigenin or carrier DMSO alone for 4 h. Then 15 μM μ-calpain was added in the presence of CaCl2. Incubation with μ-calpain effectively led to the degradation of IκBα, but only in the presence of CaCl2, as expected. At both doses of μ-calpain, the extent of degradation of the IκBα-GST protein was greater following phosphorylation by CK2. GST protein alone was resistant to treatment with 90 nM μ-calpain (Fig. 4C), which confirmed that calpain degradation of the fusion protein was mediated via the IκBα sequences. Lastly, the 3CIκBα mutant, which cannot be efficiently phosphorylated by CK2 in cellular extracts (Fig. 1A) or in vitro (Fig. 4D), was used to confirm the role of phosphorylation in enhancing degradation of IκBα by μ-calpain. The effect of CK2 treatment on the extents of degradation of wtIκBα-GST and 3CIκBα-GST by calpain were compared during a 15-min incubation in the presence of 750 μM calcium (Fig. 4E). Incubation of the 3CIκBα-GST fusion protein with CK2 had no effect on the basal extent of degradation mediated by 90 nM μ-calpain (Fig. 4E). In contrast, as shown above, CK2 phosphorylation of wtIκBα-GST enhanced the extent of degradation upon a similar treatment with μ-calpain. Thus, the enhanced rate of IκBα degradation by μ-calpain described above is dependent upon CK2 phosphorylation of sites S283, T291, and T299.

Inhibition of CK2 or calpain increase IκBα half-life in CH31 and CH12 IgM+, but not in A20 and M12 IgG+. B cells

As discussed above, the half-life of IκBα decay was previously shown to be much shorter in IgM+ vs IgG+ B cells (5). To determine whether our findings could be extended to other IgM+ B cell lines, the effects of inhibition of CK2 and calpain on the half-life of IκBα decay were assessed in CH31 and CH12 cells. A slower rate of decay of IκBα protein was similarly noted in apigenin-treated CH31 and CH12 cells compared with the control cells treated with carrier DMSO (Fig. 5A). In this and a duplicate experiment IκBα protein decayed with a t1/2 of 3.2 ± 0.95 h in the apigenin-treated vs 1.56 ± 0.1 h in the DMSO-treated control CH31 cells and a t1/2 of 5.2 ± 1.66 h in the apigenin-treated vs 2.28 ± 0.42 h in the control CH12 cells. To assess the role of calpain, cultures were next incubated in the presence of DMSO, B/E, E-64d, or calpeptin for 30 min, then cycloheximide was added, and cytoplasmic extracts were isolated at the times indicated (Fig. 5B). Addition of either calpain inhibitor significantly decreased the extent of IκBα decay, whereas degradation of p27 was unaffected (Fig. 5B). These results confirm the role of calpain in IκBα turnover in IgM+ B cells.

We next asked whether this pathway might account in part for the differences in IκBα half-life observed previously between IgG+ vs IgM+ B cells (5). To test this possibility, two IgG+ B cell lines A20 and M12, were analyzed. In agreement with previous work A20 and M12 cells displayed a longer basal half-life of decay for IκBα than the IgM+ B cell lines (Fig. 6, DMSO control lanes). Treatment with either E-64d or apigenin had little effect on IκBα turnover in A20 or M12 IgG+ B cells (Fig. 6). In contrast, addition of the proteasome inhibitor MG-132 prevented the decay of IκBα.

To assess the effects of phosphorylation by CK2 on calpain-mediated degradation, wtIκBα-GST was incubated in the absence or the presence of CK2 and then subjected to degradation with 60 or 90 nM μ-calpain in the presence of CaCl2, as described above (Fig. 4B). Alternatively, 90 nM μ-calpain was added in the absence of CaCl2. Incubation with μ-calpain effectively led to the degradation of IκBα, but only in the presence of CaCl2, as expected. At both doses of μ-calpain, the extent of degradation of the IκBα-GST protein was greater following phosphorylation by CK2. GST protein alone was resistant to treatment with 90 nM μ-calpain (Fig. 4C), which confirmed that calpain degradation of the fusion protein was mediated via the IκBα sequences. Lastly, the 3CIκBα mutant, which cannot be efficiently phosphorylated by CK2 in cellular extracts (Fig. 1A) or in vitro (Fig. 4D), was used to confirm the role of phosphorylation in enhancing degradation of IκBα by μ-calpain. The effect of CK2 treatment on the extents of degradation of wtIκBα-GST and 3CIκBα-GST by calpain were compared during a 15-min incubation in the presence of 750 μM calcium (Fig. 4E). Incubation of the 3CIκBα-GST fusion protein with CK2 had no effect on the basal extent of degradation mediated by 90 nM μ-calpain (Fig. 4E). In contrast, as shown above, CK2 phosphorylation of wtIκBα-GST enhanced the extent of degradation upon a similar treatment with μ-calpain. Thus, the enhanced rate of IκBα degradation by μ-calpain described above is dependent upon CK2 phosphorylation of sites S283, T291, and T299.

FIGURE 2. Inhibition of CK2 by apigenin decreases the rate of decay of IκBα and levels of NF-κB binding and activity in WEHI 231 cells. A, Immunoblot analysis. WEHI 231 cells were incubated with 20 μM apigenin or carrier DMSO alone for 4 h. Then 15 μg/ml cycloheximide (CHX) was added, and WCEs were prepared at the indicated times and subjected to immunoblot analysis for IκBα and IκBβ. The t1/2 values obtained in β for IκBα are given below. B, Half-life of decay. The immunoblots in A were subjected to densitometry, and the results are presented. C, EMSA. Nuclear extracts were prepared from WEHI 231 cells incubated with 20 μM apigenin or carrier DMSO alone for 8 h, and samples (3 μg) were used in EMSA with NF-κB or Oct-1 probes. D, Activity. Exponentially growing WEHI 231 cell were electroporated in duplicate with 30 μg NF-κB ele-

ment luciferase reporter DNA and 5 μg SV40 β-gal DNA to normalize for transfection efficiency. After electroporation, either 20 or 40 μM apigenin or the equivalent volume of DMSO was added, and the cultures were incubated for 8 h. Cells were harvested, and extracts were subjected to luciferase and β-gal reporter activity assays. Luciferase activity was normalized to β-gal, and results are presented as the average ± SD.
and added. After 8 h, cells were harvested and subjected to assays for luciferase.

Results are presented as the average of three independent experiments. The error bars indicate the SD.

Discussion

Here we show for the first time that basal phosphorylation of serine/threonine residues in the PEST domain of IκBα by CK2 facilitates degradation of this inhibitory protein via a calcium- and calpain-dependent pathway in IgM⁺ B cells, thereby controlling NF-κB levels and activity. This pathway was active in the WEHI 231 and CH31 immature and CH12 mature IgM⁺ B cell lines, but not in A20 and M12 IgG⁺ mature B cells. This suggests that the more rapid degradation of IκBα seen in IgM⁺ vs IgG⁺ B cells previously (5) can be attributed at least in part to this calcium- and calpain-dependent pathway. Interestingly, an important relationship was reported in Drosophila between CK2 and the stability of Cactus, the selective inhibitor of the NF-κB/Rel-related Dorsal protein, which is responsible for dorsal patterning (26). In particular, serine to alanine mutations in the PEST domain of Cactus prevented CK2 phosphorylation, increasing the stability of Cactus while not affecting its ability to bind to Rel. In mammalian cells experiments have similarly shown that phosphorylation by CK2 of Ser²⁸³, Thr²⁹¹, Ser²⁹³, and Thr²⁹⁵ in the C-terminus plays a significant role in the regulation of IκBα stability (11–15). Our studies provide data on the mechanism by showing that CK2 phosphorylation of serine/threonine residues in the PEST domain promotes degradation of IκBα via calpain.
While a majority of studies indicate that IκBα degradation during signal-induced activation of NF-κB is mediated via a ubiquitin-proteasome pathway (7, 27), several lines of evidence suggest that alternative pathways operate under basal as well as inducible situations. As discussed above, a proteasome-independent proteolytic pathway was reported to mediate basal degradation of IκBα in WEHI 231 cells and other primary B cells (9, 10). Turnover of IκBα mutated in the PEST domain, but not at Ser32/36, was found to occur with the reduced kinetics following induction of oxidative stress upon treatment with H₂O₂ (8). Furthermore, TNF-α-inducible proteolysis of IκBα in human liver and airway epithelial cells was mediated by cytosolic m-calpain (28). Overexpression of the intracellular calpain inhibitor calpastatin was shown to block both basal and silica-induced NF-κB activation in human bronchial epithelial cell lines (29, 30). Calpain inhibitor I was also shown to inhibit NO synthesis by blocking IκBα degradation (31). Finally, mice deficient in the skeletal muscle-specific calpain 3 were found to display myoneural apoptosis and profound perturbation of the IκBα/NF-κB pathway typical of limb-girdle muscular dystrophy type 2A (32). These findings solidify the initial in vitro observation made by Shumway et al. (15), which indicated that IκBα is a potential proteolytic target of μ-calpain, and that the PEST domain of this protein is necessary and sufficient for this process to occur. In our study we extend this observation by linking phosphorylation of IκBα-GST by recombinant CK2 with its degradation via μ-calpain. This enhanced calpain-mediated degradation following phosphorylation of CK2 in the PEST domain probably contributes to the observed intracellular role of calpain in control of the intrinsic stability of IκBα.

Elevated CK2α expression in T cells of transgenic mice led to development of T cell lymphomas (33). More recently, transgenic overexpression of CK2α in the mammary gland was found to lead to breast tumors in mice, and cells derived from these mammary tumors contained functional NF-κB activity (34). Consistent with these observations, human breast cancer cell lines and multiple primary breast cancer specimens displayed constitutive CK2 activity; inhibition of this activity in culture with either apigenin or emodin resulted in reduced NK-κB binding and activity (35). The potential role of CK2 in the regulation of aberrant NF-κB expression that typifies many tumors will require further investigation.

To date, two degradation pathways for IκBα have been identified: proteasome and calpain. Miyamoto (9) reported that the pathway in WEHI 231 cells was mediated exclusively via calcium and calpain, rather than the proteasome. Interestingly, we observed that...
treatment of WEHI 231 cells with MG-132, a selective proteasome inhibitor, also reduced the extent of basal turnover of IkBα (t1/2 = 114 vs 55 min in control DMSO-treated cells) and of control p27Kip1 protein as in CH31 and CH12 cells (data not shown). These results suggest that both calpain- and proteasome-mediated degradation may contribute to the rapid IkBα turnover seen in IgM+ B cells (5). The differences between the previous studies (9) and our findings with respect to WEHI 231 cells probably represent clonal variations between the lines. Thus, while our experiments make a strong case for phosphorylation by CK2 playing a role in degradation of IkBα by μ-calpain, they do not rule out the possibility that CK2 also plays a role in proteasome-mediated degradation in these cells. However, the data do indicate that CK2 phosphorylation does not play a role in proteasome-mediated degradation in IgG+ cells, since apigenin had no effect on IkBα decay in either M12 or A20 cells.

In summary, our findings begin to provide a mechanism for the previously observed role of CK2 in half-life of decay of IkBα protein and for the differences in control of NF-κB in IgM+ vs IgG+ B cells. Interestingly, CK2 was recently also implicated in signal-dependent (e.g., TNF-α) degradation of IkBα in association with NF-κB (36). The reason why cells bearing different isotypes of B cell Ag receptor use different mechanisms of IkBα turnover is not clear. It would be interesting to determine whether developmental regulation of either the level or the activity of CK2 or calpain exists in B cells. Furthermore, engagement of the surface IgM B cell Ag receptor on mature vs immature B cells leads to dramatically different effects on turnover of IkBα proteins and NF-κB levels (16, 18, 37, 38). Whether the effects on IkBα turnover are mediated via CK2 or calpain is under investigation.

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