The Serine/Threonine Phosphatase Inhibitor Calyculin A Induces Rapid Degradation of IκBβ

REQUIREMENT OF BOTH THE N- AND C-TERMINAL SEQUENCES∗

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Signal-initiated activation of the transcription factor NF-κB is mediated through proteolysis of its cytoplasmic inhibitory proteins IκBa and IκBβ. While most NF-κB inducers trigger the degradation of IκBa, only certain stimuli are able to induce the degradation of IκBβ. The degradation of IκBa is targeted by its site-specific phosphorylations, although the mechanism underlying the degradation of IκBβ remains elusive. In the present study, we have analyzed the effect of phosphatase inhibitors on the proteolysis of IκBβ. We show that the serine/threonine phosphatase inhibitor calyculin A induces the hyperphosphorylation and subsequent degradation of IκBβ in both human Jurkat T cells and the murine 70Z-3 preB cells, which is associated with the nuclear expression of active NF-κB. The calyculin A-mediated degradation of IκBβ is further enhanced by the cytokine tumor necrosis factor-α (TNF-α), although TNF-α alone is unable to induce the degradation of IκBβ.

The NF-κB/Rel family of transcription factors play a pivotal role in the regulation of various cellular genes involved in the immediate early processes of immune, acute phase, and inflammatory responses (1, 2). In addition, these cellular factors have also been implicated in the transcriptional activation of certain human viruses, most notably the type 1 human immune deficiency virus (3–7). The mammalian NF-κB/Rel family is composed of at least five structurally related DNA-binding proteins, including p50, p52, RelA, RelB, and c-Rel, which bind to a target DNA sequence (κB) as various heterodimers or homodimers (reviewed in Siebenlist et al. (8)). In most cell types, including resting T cells, the NF-κB/Rel proteins are sequestered in the cytoplasmic compartment by physical association with inhibitory proteins that are characteristic of the presence of various numbers of ankyrin-like repeats (reviewed in Verma et al. (9)). The major cytoplasmic inhibitors include IκBa (10, 11), IκBβ (12), and the precursor proteins of p50 and p52 (9). The IκB molecules appear to bind to and mask the nuclear localization signal of NF-κB/Rel, thereby preventing the nuclear translocation of these transcription factors (13–16).

The latent cytoplasmic NF-κB/Rel complexes can be activated by a variety of cellular stimuli, including the mitogen phorbol esters, cytokines such as tumor necrosis factor-α (TNF-α), and interleukin-1, the bacterial component lipopolysaccharide, serine/threonine phosphatase inhibitors such as okadaic acid and calyculin A, and the Tax protein from the type I human T cell leukemia virus (HTLV-I) (8, 17). Activation of NF-κB by these various inducers involves phosphorylation of IκBa at serines 32 and 36 (18–23), which in turn targets this inhibitory protein for ubiquitination and proteasome-mediated proteolysis (24, 25). Since the IκBa gene is positively regulated by the NF-κB/Rel factors, the depleted IκBa protein pool can be rapidly replenished through de novo protein synthesis following the activation of NF-κB/Rel (26–31). Thus, IκBa regulates the transient nuclear expression of NF-κB/Rel. Unlike IκBa, IκBβ appears to respond to only certain cellular stimuli, such as lipopolysaccharide, interleukin-1, and Tax, that are known to induce sustained nuclear expression of NF-κB/Rel (12, 32, 33). The depleted IκBβ protein is not immediately resynthesized, which is likely the molecular basis of persistent activation of NF-κB/Rel.

The molecular mechanism underlying the differential signal responses between IκBa and IκBβ remains elusive. Although IκBβ contains two N-terminal serines (serines 19 and 23) that are homologous to the inducible phosphorylation sites present in IκBa. Furthermore, the C-terminal 51 amino acid residues, which are rich in serines and aspartic acids, are also required for the inducible degradation of IκBβ. These results suggest that the degradation signal of IκBβ may be controlled by the opposing actions of protein kinases and phosphatases and that both the N- and C-terminal sequences of IκBβ are required for the inducible degradation of this NF-κB inhibitor.

The abbreviations used are: TNF-α, tumor necrosis factor-α; HTLV-I, type I human T cell leukemia virus; EMSA, electrophoresis mobility shift assay; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.

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Induction of IκBβ Phosphorylation and Degradation

MATERIALS AND METHODS

Cell Culture and Reagents—Jurkat T cells (ATCC) and Jurkat cells expressing the SV40 large T antigen (Jurkat Tag) (34) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and antibiotics. Murine 70Z/3 pre-B cells (ATCC) were maintained in the same medium supplemented with 50 μg/μl β-mercaptoethanol. The serine/threonine phosphatase inhibitor calyculin A was purchased from LC Laboratories (Woburn, MA). The proteasome inhibitor MG132 was purchased from ProScript, Inc. (Cambridge, MA). The antibody against the influenza hemagglutinin (HA) epitope tag (anti-HA) was obtained from Boehringer Mannheim. Anti-IκB (C-20) was purchased from Santa Cruz Biotechnology, Inc.

Plasmid Constructs and Transient Transfection—The wild type of pCMV4HA, IκBβ was constructed by cloning the IκBβ cDNA (kindly provided by Dr. Sankar Ghosh, Yale University) (12) into a modified pCMV4 expression vector, pCMV4HA (22), downstream of three copies of the HA epitope tag (YPYDVPDYA). IκBβ was also generated by site-directed mutagenesis. The C-terminal truncation mutant {IκBβ(1–208)} was constructed by introducing a stop codon after codon 308 of the wild type IκBβ by restriction digestion (using HindIII), DNA polymerase (Klenow fragment) fill in, and religation. Jurkat Tag cells (5 × 10⁶) were transfected using DEAE-dextran (35) with the indicated amounts of IκBβ expression vectors. Between 40 and 48 h post-transfection, the cells were incubated with calyculin A (25 nM) and TNF-α (10 ng/ml) for the indicated time periods and then subjected to whole extract preparation and immunoblotting analyses as described below.

Immunoblotting and Electrophoresis Mobility Shift Assay (EMSA)—Jurkat cells, 70Z/3 pre-B cells, or transiently transfected Jurkat-Tag cells were stimulated with the indicated inducers and then collected by centrifugation at 800 × g for 5 min. Whole cell and subcellular extracts were prepared as described previously (36, 37). For immunoblotting analyses, whole cell extracts (∼15 μg) were fractionated by reducing 8.75% SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and then analyzed for immunoreactivity with the indicated primary antibodies using an enhanced chemiluminescence detection system (ECL; Amersham Corp.). For in vitro phosphorylation treatment, the extracts were incubated with 20 units of calf intestinal alkaline phosphatase at 35 °C for 30 min prior to immunoblotting analysis. EMSAs were performed by incubating the nuclear extracts (∼3 μg) with a [32P]-radiolabeled high-affinity palindromic xB probe, xB-pd (coding strand sequence was 5′-CAACGGCAGGGGAATTCCCCTCTCCTTCCTTCCT-3′) followed by resolving the DNA-protein complexes on native 5% polyacrylamide gels (38).

RESULTS

Calyculin A Induces the Rapid Phosphorylation and Degradation of IκBβ and the Concurrent Nuclear DNA Binding Activity of NF-κB in Both Human Jurkat T Cells and Murine 70Z/3 Pre-B Cells—To investigate the effect of the phosphatase inhibitors on the fate of IκBβ, Jurkat T cells were incubated with calyculin A for different time periods followed by analysis of the IκBβ protein by immunoblotting (Fig. 1A, upper panel). In untreated cells, a single 45-kDa form of IκBβ was detected with an IκBβ-specific antiserum (Fig. 1A, upper panel, lane 1). Incubation of the cells with calyculin A (25 nM) for 15 min led to a marked loss of the preexisting IκBβ protein (lane 3), which persisted until at least 1 h after calyculin A stimulation (lanes 3–5). The loss of IκBβ was apparently due to its proteolysis as this effect of calyculin A was blocked by a potent proteasome inhibitor, MG132 (lane 7), known to inhibit the degradation of IκBα (24, 39). Parallel EMSA revealed that degradation of IκBβ was associated with the appearance of the NF-κB DNA binding activity in the nucleus (Fig. 1A, lower panel). We noticed that the IκBβ isolated from cells treated with calyculin A migrated more slowly on the SDS-polyacrylamide gel compared to the basal form of IκBβ (Fig. 1A, compare lanes 1 and 6 with lanes 2–5 and 7). To examine whether the slower migration of IκBβ might be due to its phosphorylation, the cell extract was incubated with calf intestine alkaline phosphatase before being subjected to immunoblotting (Fig. 1B).

After calf intestine alkaline phosphatase treatment, the more slowly migrating IκBβ species from calyculin A-treated cells (lane 2) was completely converted to a faster-migrating IκBβ (Fig. 1B, lane 3), thus suggesting that the slower migration of IκBβ in calyculin A-treated cells was due to its phosphorylation. Interestingly, the in vitro dephosphorylated form of IκBβ (lane 3) migrated slightly faster than the basal form present in untreated cells (lane 1). This result suggests that as seen with murine 70Z/3 pre-B cells (40), IκBβ is preexisting in a basally phosphorylated form in untreated Jurkat T cells. This basal form of IκBβ appears to become hyperphosphorylated when the cells are treated with calyculin A. Of note, the hyperphosphorylation of IκBβ appeared to precede its degradation since inhibition of IκBβ degradation by MG132 led to the accumulation of the more slowly migrating hyperphosphorylated IκBβ (Fig. 1A, upper panel, lane 7).

To examine whether the effect of calyculin A on IκBβ could be recapitulated in other cell types, murine 70Z/3 pre-B cells were subjected to the calyculin A treatment. In untreated 70Z/3 cells, IκBβ is preexisting in two forms that migrate with slightly different rates on SDS-PAGE (Fig. 1C, upper panel, lane 1). The more slowly migrating band was apparently the phosphorylated form of IκBβ since it was converted to the more rapidly migrating form after in vitro incubation with calf intestinal alkaline phosphatase (data not shown) (40). More importantly, incubation of the 70Z/3 cells with calyculin A led to the rapid degradation of the preexisting IκBβ proteins (Fig. 1C, lanes 2–5, upper panel). Furthermore, as observed in Jurkat T cells, the degradation of IκBβ was preceded by the appearance of the more slowly migrating hyperphosphorylated IκBβ (lanes 2–5).

Together, these results suggest that the serine/threonine
phosphatase inhibitor calcyclin A is able to induce the dephosphorylation of IκBα in both Jurkat T cells and 70Z/3 pre-B cells and that the degradation of IκBα is preceded by its hyperphosphorylation.

TNF-α Promotes Calyculin A-Induced Degradation of IκBα—Prior studies have demonstrated that the TNF-α-elicted celllar activation signal is insufficient to induce the degradation of IκBα, although this signal induces the degradation of IκBα (12). To investigate whether the TNF-α-mediated signal could synergize with the phosphatase inhibitor in the degradation of IκBα, we examined the effect of TNF-α on calcyclin A-mediated degradation of IκBα. As previously reported, incubation of Jurkat T cells with TNF-α alone was inefficient in the induction of IκBα degradation (Fig. 2, lanes 4 and 5). However, when the cells were treated with TNF-α together with calcyclin A, significant IκBα degradation could be detected as early as 5 min after cellular stimulation (lane 6), and the entire intracellular pool of IκBα was almost completely depleted at 30 min post-stimulation (lane 7). Consistent with the results shown in Fig. 1A, calcyclin A alone induced the degradation of IκBβ (lanes 2 and 3); however, the kinetics of IκBβ degradation in these cells was slower compared to that detected in cells costimulated with TNF-α and calcyclin A (compare lanes 2 and 3 with lanes 6 and 7).

Both the N- and C-terminal Sequences Are Required for Degradation of IκBβ—To further explore the biochemical mechanism underlying the induction of IκBβ degradation by calcyclin A and TNF-α, studies were performed to examine the sequences required for the inducible degradation of IκBβ. For these studies, cDNA expression vectors encoding HA-tagged wild type or mutant IκBβ were transfected into Jurkat T cells, and the inducible degradation of these IκBβ proteins was analyzed by immunoblotting using an anti-HA antibody. The exogenously transfected wild type IκBβ expressed as two forms with slightly different mobilities on SDS-PAGE (Fig. 3A, lane 2). As seen in 70Z/3 cells, the differential mobility of these two forms of IκBβ appeared to be due to different levels of phosphorylation as demonstrated by in vitro calf intestinal alkaline phosphatase assays (data not shown). Stimulation of the transfectants with calcyclin A and TNF-α led to the gradual depletion of the ectopic IκBβ (lanes 3–5). Thus, as seen with its endogenous counterpart, the transfected HA-tagged IκBβ could be degraded in response to cellular stimulation. Deletion of an N-terminal region (amino acids 5–27) covering two potential phosphorylation sites (serines 19 and 23) did not influence the basal phosphorylation of IκBβ, since both the slow and fast migrating bands were detected in cells transfected with this mutant (Fig. 3B, lane 4). However, this IκBβ deletion mutant failed to be degraded following cellular stimulation with calcyclin A and TNF-α (lanes 4–6). To examine whether serines 19 and 23 were important for the degradation of IκBβ, an IκBβ mutant bearing mutations at these sites was tested in the degradation assay. As expected, mutation of these two serines to alanines significantly inhibited the degradation of IκBα (lanes 1–3). We then examined the potential role of the C-terminal sequences in the degradation of IκBβ. In this regard, the C-terminal portion of IκBβ is rich in serines and aspartic acids (12) and has recently been shown to contain the sites for constitutive phosphorylation (41). Consistent with this recent study, an IκBβ mutant lacking the C-terminal 51 amino acids (IκBβ(1–308)) migrated on SDS-PAGE as a single band (lane 7), indicating the lack of constitutive phosphorylation. More importantly, deletion of the C-terminal acidic sequences markedly inhibited the degradation of IκBβ (lanes 8 and 9). Thus, degradation of IκBβ induced by calcyclin A and TNF-α requires both the N-terminal potential phosphorylation sites and the C-terminal sequences.

DISCUSSION

The nuclear expression and biological function of the NF-κB transcription factor is tightly regulated through its cytoplasmic retention by ankyrin-rich inhibitors, including IκBo and IκBβ (12–16). Activation of NF-κB by various cellular stimuli involves the site-specific phosphorylation and subsequent proteolytic degradation of IκBo, which is associated with the transient nuclear expression of the liberated NF-κB factors (18–21). However, activation of the IκBα-sequestered NF-κB pool is triggered by only certain cellular stimuli, which normally induce persistent NF-κB activation, such as lipopolysaccharide and interleukin-1 (12) and the HTLV-I Tax protein (32, 33). It remains elusive why the two types of IκB molecules differentially respond to the cellular activation signals. Although IκBα contains two N-terminal serines (Ser19/Ser23) that are homologous to the inducible phosphorylation sites of IκBo, it is not clear whether these sites are phosphorylated in response to cellular stimulation.

In the present study, we have demonstrated that the serine/threonine phosphatase inhibitor calcyclin A is able to induce the phosphorylation and degradation of IκBβ. This finding supports our model that phosphorylation current triggers the polyubiquitination of IκBα. However, from our current study, we cannot conclude that the two N-terminal serines (serines 19 and 23) are phosphorylated. To directly address this question, phosphopeptide analyses are necessary. It is clear, though, that site-directed mutagenesis of these two serines to alanines markedly attenuates degradation by calcyclin A (Fig. 3) and...
other inducers such as Tax, TNF-α (in HeLa cells), and PMA/anti-CD28 (21, 33, 42). Interestingly, we observed basal phosphorylation of IκBβ in both Jurkat and 70Z/3 cells (Fig. 1); however, this appears to involve phosphorylation of sites within the C-terminal region (Fig. 3) (41).

We have also demonstrated that in Jurkat T cells, TNF-α is in synergy with calcineurin A as a result of NF-κB degradation. In agreement with a previous study (12), in these leukemic T cells, TNF-α alone is not sufficient to induce the degradation of IκBβ (Fig. 2), although it is efficient in the induction of IκBO degradation (27). These results suggest that the signals required for triggering the degradation of IκBβ are more stringent than those for the degradation of IκBα. One possibility is that the N-terminal region of IκBβ is not as efficient a substrate for phosphorylation as the homologous region of IκBα. Phosphorylation of IκBβ thus would require more potent signals which presumably would result in more vigorous kinase activity. Phosphatase inhibitors would act in this manner by leaving kinase activity virtually unopposed by any regulatory cellular phosphatases.

As seen with IκBα, the C-terminal region of IκBβ is rich in prolines, serines, and acidic amino acids. Such a sequence, known as PEST, has been proposed to be involved in the rapid turnover of certain proteins (43). The PEST sequence appears to be dispensable for the inducible degradation of IκBα, although this C-terminal region may regulate the constitutive turnover of IκBα (22, 44, 45). However, the PEST sequence is likely required for the inducible degradation of IκBβ since deletion of this region renders IκBβ nonresponsive to the potent degradation signals initiated by calcineurin A together with TNF-α (Fig. 3B). A recent study (41) suggests that phosphorylation of two serines within the PEST region is critical for the interaction of IκBβ with the c-Rel protooncoprotein. However, it is unclear whether the basal phosphorylation at the C terminus of IκBβ plays a role in regulation of its inducible degradation, although a C-terminal truncation mutant of IκBβ lacking these phosphorylation sites becomes unresponsive to the degradation signals (Fig. 3B). Studies are in progress to determine possible functions of the IκBβ C-terminal PEST domain and to map specific amino acids in the C terminus required for inducible degradation.

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