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Role of Unphosphorylated, Newly Synthesized IκBβ in Persistent Activation of NF-κB

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Stimulation with inducers that cause persistent activation of NF-κB results in the degradation of the NF-κB inhibitors, IκBα and IκBβ. Despite the rapid resynthesis and accumulation of IκBα, NF-κB remains induced under these conditions. We now report that IκBβ is also resynthesized in stimulated cells and appears as an unphosphorylated protein. The unphosphorylated IκBβ forms a stable complex with NF-κB in the cytosol; however, this binding fails to mask the nuclear localization signal and DNA binding domain on NF-κB, and the IκBβ–NF-κB complex enters the nucleus. It appears therefore that during prolonged stimulation, IκBβ functions as a chaperone for NF-κB by protecting it from IκBα and allowing it to be transported to the nucleus.

The transcription factor NF-κB is a ubiquitously expressed transcription factor that plays an important role in the inducible expression of a large number of cellular and viral genes (3, 15, 24). In the majority of cells, NF-κB exists in an inactive form in the cytoplasm by being bound to the inhibitory protein IκBα or IκBβ (2, 25, 26). Treatment of cells with various inducers results in the degradation of IκB proteins, thus releasing the bound NF-κB, which translocates to the nucleus and upregulates gene expression (4, 8, 9, 13, 21–23, 25). IκBα is degraded by all of the known inducers of NF-κB, whereas IκBβ is degraded only when cells are stimulated with inducers such as lipopolysaccharide (LPS) and interleukin-1 (IL-1) which cause persistent activation of NF-κB. In either case, active NF-κB causes an upregulation of IκBα mRNA levels as a result of the presence of NF-κB sites in the IκBα promoter (10, 16). The newly synthesized IκBα helps to terminate the NF-κB response by resequestering NF-κB. However, in persistent activation by LPS or IL-1, some of the NF-κB appears to be insensitive to newly made IκBα (25). It is unknown, however, how this pool of NF-κB escapes inhibition by the newly made IκBα and why only inducers that affect IκBβ are able to cause persistent activation.

We report here that following degradation of the initial pool of IκB-β in response to inducers such as LPS or IL-1, newly synthesized IκBβ accumulates as an unphosphorylated protein that forms a stable complex with a portion of NF-κB and prevents it from binding to newly synthesized IκBα. The IκBβ in its unphosphorylated state differs from basally phosphorylated IκBβ, as it is unable to mask the nuclear localization signal (NLS) and the DNA binding domain of NF-κB. Therefore, the NF-κB bound to unphosphorylated IκBβ can enter the nucleus and bind to DNA. Hence, during persistent activation of NF-κB, the newly synthesized, unphosphorylated IκBβ plays a chaperone-like role, by binding to a portion of newly synthesized NF-κB and allowing it to be transported to the nucleus.

MATERIALS AND METHODS

Immunoblot analysis. Immunoblot analysis was generally carried out with 25 to 30 μg of extract. Following fractionation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), the proteins were electrophoretically transferred to polyvinylidene difluoride membranes. For detecting slightly faster mobility of the unphosphorylated protein, SDS-PAGE was carried out on 11-cm-long Hoefer minigels instead of the 7-cm-long gels used normally. Following transfer, the membrane was blocked with 5% BLOTTO, and the primary antibodies (IκBβ antiserum at 1:1,000 dilution; IκBα antibody [Santa Cruz Biotechnology] and p65 antibody [Biomol] at 1:1,000 dilution) were added in BLOTTO. Subsequent washes and incubation with the secondary antibody were done in Tween–Tris-buffered saline (TTBS). Proteins detected by the primary antibody were visualized by carrying out an enhanced chemiluminescence (ECL) assay using (Amersham) ECL reagents and exposure to film.

Preparation of cytosolic and nuclear extracts. Approximately 2×10^7 cells were used for each time point. The cells were pelleted by low-speed centrifugation, washed with phosphate-buffered saline (PBS), and then resuspended in 200 μl of buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 10 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mM NaF, 1 mM β-glycerophosphate, cocktail of protease inhibitors [leupeptin, pepstatin, aprotinin, and phenylmethylsulfonyl fluoride). After the cells were allowed to swell, 5 μl of 0.5% Nonidet P-40 (NP-40) was added and the cells were gently vortexed for 10 s. Following centrifugation, the pelleted nuclei were washed with buffer A, resuspended in 50 μl of buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mM NaF, 1 mM β-glycerophosphate, cocktail of protease inhibitors) and shaken for 15 min at 4°C. The extract was centrifuged, and glycerol was added to 5%. The cytoplasmic fraction obtained after the low-speed centrifugation was centrifuged at $100,000 \times g$ for 1 h, and the supernatant was adjusted to 100 mM NaCl–5% glycerol.

Detection of DNA-binding proteins by using biotinylated oligonucleotides. Approximately 5×10^7 cells were used for each time point. Nuclear and cytosolic extracts were prepared from unstimulated and LPS-stimulated cells, using the protocol described above. The extracts were precleared by mixing with insoluble protein A-Sepharose, washed with PBS, and then precleared with streptavidin-agarose. The precleared extracts were then incubated with the biotinylated and radioactively labeled κB probe in the presence of excess poly(dI-dC) as the competitor. Then the streptavidin-agarose beads were added, and the mixture was incubated at 4°C for 60 min. The beads were then spun down and washed extensively four times with a buffer containing 50 mM Tris (pH 7.9), 150 mM NaCl, 0.1 mM EDTA, 0.5% NP-40, and 10% glycerol. After the final wash, the beads were resuspended in 50 ml of SDS sample buffer and boiled, and the supernatant was analyzed by SDS-PAGE and immunoblotting.

Purification of IκBβ overexpressed in COS cells. The IκBβ cDNA was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and transiently transfected into COS cells along with the p65 cDNA in the pcDNA3 expression vector, using Lipofectamine (GIBCO-BRL). Under optimum conditions, transfection efficiencies averaging 75% are obtained. Cotransfection with p65 is necessary to obtain high levels of accumulation of IκBβ. The cells were harvested 48 h after transfection, and extracts were made by using NP-40 lysis. The extract was first batch purified by loading it on a DEAE-Sephacel column at 400 mM NaCl. The flowthrough (which contains the majority of the cellular proteins) was adjusted to 100 mM NaCl and then treated with 0.8% deoxycholate to dissociate IκBβ from NF-κB before being loaded onto another DEAE-Sephacel column. The majority of the NF-κB elutes in the flowthrough, while the IκBβ remains bound to the column. The bound protein was then eluted with a gradient from 100 to 600 mM NaCl. The fractions were assayed for activity by using purified NF-κB and electrophoretic mobility shift assay (EMSA), and the peak fractions of activity were pooled. These fractions were then adjusted to 100 mM NaCl and loaded onto a fast protein liquid chromatography (FPLC) Mono Q

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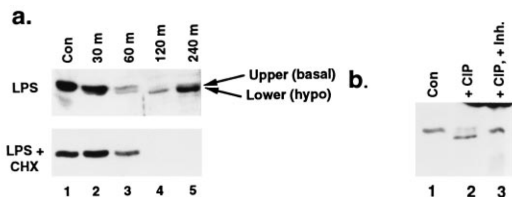

FIG. 1. Persistent activation by LPS results in the accumulation of unphosphorylated IκBβ. (a) Equal numbers of 70Z/3 cells were untreated (control [Con]) or treated with LPS (10 μg/ml) or LPS and cycloheximide (CHX; 10 μg/ml) for the indicated periods of time (minutes [m]). Cytosolic extracts were prepared, fractionated by SDS-PAGE (10% gel), and subjected to immunoblot analysis with the IκBβ antibody, and the bound antibodies were detected by ECL. The faster-migrating forms of IκBβ in lanes 4 and 5 are of significantly lower abundance and require long exposures on ECL for visualization. The upper, basally phosphorylated and the lower, hypophosphorylated (hypo) form also migrate very close to one another and are difficult to resolve on standard-length gels. Addition of cycloheximide blocks the appearance of the lower form of IκBβ (lanes 3 to 5). (b) Thirty micrograms of the cytosolic extract from uninduced 70Z/3 cells was incubated with calf intestine alkaline phosphatase (CIP). Lanes: 1, no phosphatase; 2, 24 U of alkaline phosphatase; 3, 24 U of alkaline phosphatase and a mixture of phosphatase inhibitors (Inh.; 10 mM β-glycerophosphate and 10 mM sodium fluoride). The reactions were carried out at 30°C for 30 min, and then the proteins were fractionated by SDS-PAGE and immunoblotted with the IκBβ antibody.

column. The activity (assayed by EMSA) eluted in three fractions, designated early, middle, and late.

Antibodies. The antibodies used were as follows: IκB-α, a polyclonal antipeptide antibody from N. Rice; IκBβ and p50, polyclonal antibodies generated in this laboratory against the recombinant proteins; p65 NLS, a monoclonal antibody from Boehringer Mannheim; and p65 C-terminal peptide, a polyclonal antipeptide antibody from Biomol. The protocols for immunoblotting and immunoprecipitations were as described previously (25).

RESULTS

Newly synthesized IκBβ accumulates in an unphosphorylated form. Following stimulation of 70Z/3 pre-B cells with LPS, both IκBα and IκBβ proteins are degraded, and their levels decrease dramatically. If stimulation is continued (>60 min), the level of IκBα begins to increase until it reaches the initial level, but in contrast, newly synthesized IκBβ accumulates to levels significantly lower than that present initially. Upon exposure of the ECL immunoblots for longer periods and by using longer SDS-polyacrylamide gels for immunoblotting analyses, it became apparent that the newly synthesized IκBβ protein in LPS-stimulated cells was a slightly faster migrating species (Fig. 1a, top, lanes 3 to 5). Addition of cycloheximide prevented the appearance of the faster-migrating IκBβ at later time points, proving that new protein synthesis was required (Fig. 1a, bottom, lanes 3 to 5). We presumed that the altered migration of newly synthesized IκBβ protein in LPS-stimulated cells was most likely due to some kind of posttranslational modification. To determine if the modification was phosphorylation, IκBβ from unstimulated 70Z/3 cells was treated with alkaline phosphatase. Treatment with phosphatase caused the protein to migrate faster (Fig. 1b, lane 2), and this effect was blocked when phosphatase inhibitors were included (lane 3). We have also observed that whereas ^{32}P-labeled IκBβ can be immunoprecipitated primarily from unstimulated cells, ^{35}S-labeled IκBβ can be immunoprecipitated from both unstimulated and stimulated cells (data not shown). Hence, in uninduced cells, IκBβ existed in a basally phosphorylated form, but following LPS stimulation, the new IκBβ that is synthesized appeared to be an unphosphorylated protein.

Unphosphorylated, newly synthesized IκBβ forms a stable complex with NF-κB. We then examined whether the unphosphorylated IκBβ protein observed upon prolonged LPS stimulation was in a complex with NF-κB. IκBβ was immunoprecipitated from both unstimulated cells and cells stimulated with LPS for 4 h, and the immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with an anti-p65 antibody. p65 was coimmunoprecipitated with IκBβ in both instances, suggesting that the newly synthesized, unphosphorylated IκBβ formed a complex with NF-κB (Fig. 2a, lanes 1 and 3). To prove that p65 in LPS-stimulated cells was bound to the unphosphorylated form of IκBβ, we performed a reciprocal experiment in which we immunoprecipitated p65 from unstimulated and LPS-stimulated cells. The immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with an IκBβ antibody. As seen in Fig. 2b (lane 2), p65 was associated with the faster-migrating form of IκBβ in LPS-stimulated cells.

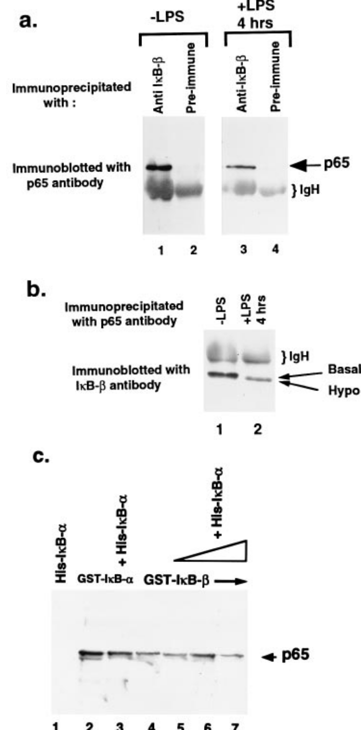

FIG. 2. Unphosphorylated IκBβ forms a stable complex with NF-κB. (a) Approximately 10^7 70Z/3 cells were either untreated or treated with LPS (10 μg/ml) for 4 h. The cells were lysed in an NP-40-containing buffer and immunoprecipitated with either the IκBβ antiserum or the corresponding preimmune serum. The immunoprecipitates were boiled with SDS-containing buffer, fractionated by SDS-PAGE, electrophoretically transferred to a polyvinylidene difluoride membrane, and immunoblotted with an affinity-purified antibody against p65. IgH, immunoglobulin heavy chain. (b) Experiment similar to that shown in panel a except that the immunoprecipitation was carried out with a p65 antibody and the immunoblotting was done with the IκBβ antibody. Hypo, hypophosphorylated form. (c) In vitro-translated, ^{35}S-labeled NF-κB p65 subunit was incubated with six-His-tagged IκBα (lane 1), GST-IκBα (lanes 2 and 3), and GST-IκBβ (lanes 4 to 7) for 5 min at room temperature. The amount of GST-IκB proteins used was sufficient to precipitate all of the NF-κB p65, indicating that the IκB proteins were in a complex with NF-κB. Then recombinant six-His-tagged IκBα was added in various amounts to the preformed GST–IκBα/β–NF-κB complexes and further incubated for 10 min. Lane 3, twice the amount of GST-IκBα used; lanes 5 to 7, 0.5, 1, and 2 times the amount of GST-IκBβ used. GST-IκBβ was then precipitated by using glutathione-agarose beads, and the amount of ^{35}S-labeled p65 coprecipitated was analyzed by SDS-PAGE fractionation of the precipitates and fluorography. The amount of recombinant protein used in each assay was quantitated by SDS-PAGE and Coomassie blue staining (b).

To determine whether unphosphorylated IκBβ bound to NF-κB could potentially shield NF-κB from newly synthesized

IκBα, we examined the stability of NF-κB–IκBβ complexes in the presence of excess IκBα. In vitro-translated, ^{35}S-labeled NF-κB p65 was incubated with glutathione *S*-transferase (GST)–IκBα or GST-IκBβ protein (bacterially produced and hence unphosphorylated) to form a GST–IκB–NF-κB p65 complex (Fig. 2c, lanes 2 and 4). Then six-histidine-tagged IκBα, produced in bacteria, was added to the reaction to determine whether IκBα could displace IκBα or IκBβ already bound to p65 (Fig. 2c). The GST-tagged IκB proteins were precipitated with glutathione-agarose beads, and the amount of p65 that was coprecipitated was determined by SDS-PAGE. The results indicated that the complex of unphosphorylated IκBβ and NF-κB p65 was quite stable because levels of IκBα that were at least twofold greater than the amount of GST-IκBβ (compare lane 7 with lane 4) were only partially effective in disrupting the complex. Therefore, unphosphorylated IκBβ forms a stable complex with NF-κB. Interestingly, free His-tagged IκBα was unable to completely displace the GST-IκBα that was already prebound to NF-κB (lanes 2 and 3), indicating that IκBα bound to NF-κB is no longer freely exchangeable, probably because of a change in conformation resulting in an increase in the stability of the interaction between the two proteins.

Unphosphorylated IκBβ binds to NF-κB but is not able to block DNA binding. Earlier reports had demonstrated that purified IκBβ when dephosphorylated with alkaline phosphatase was unable to inhibit NF-κB DNA binding activity, suggesting that dephosphorylated IκBβ did not interact with NF-κB (14, 17). In conflict with this interpretation, however, was our observation that bacterially expressed GST-IκBβ was able to interact with p65 and c-Rel, although our study did not test the ability of IκBβ to inhibit DNA binding by NF-κB (25). To determine whether there was a difference in the abilities of basally phosphorylated and dephosphorylated IκBβ to inhibit NF-κB DNA binding, bacterially produced IκBβ was compared with IκBβ translated in rabbit reticulocyte lysates. When GST-IκBα and GST-IκBβ proteins (Fig. 3a, lanes 2 and 3) were used in a standard NF-κB DNA binding inhibition assay, we observed that bacterially expressed (and therefore unphosphorylated) IκBβ was unable to efficiently inhibit DNA binding by NF-κB (lanes 5 to 7). The presence of IκBβ in the DNA-protein complex was apparent from the slower mobility relative to the migration of the DNA-protein complex containing NF-κB alone (compare lane 1 with lane 5). In addition, the complex of GST-IκBβ and NF-κB bound to DNA could be supershifted by an antibody against GST (Fig. 3d). To eliminate the possibility that the results obtained were influenced by

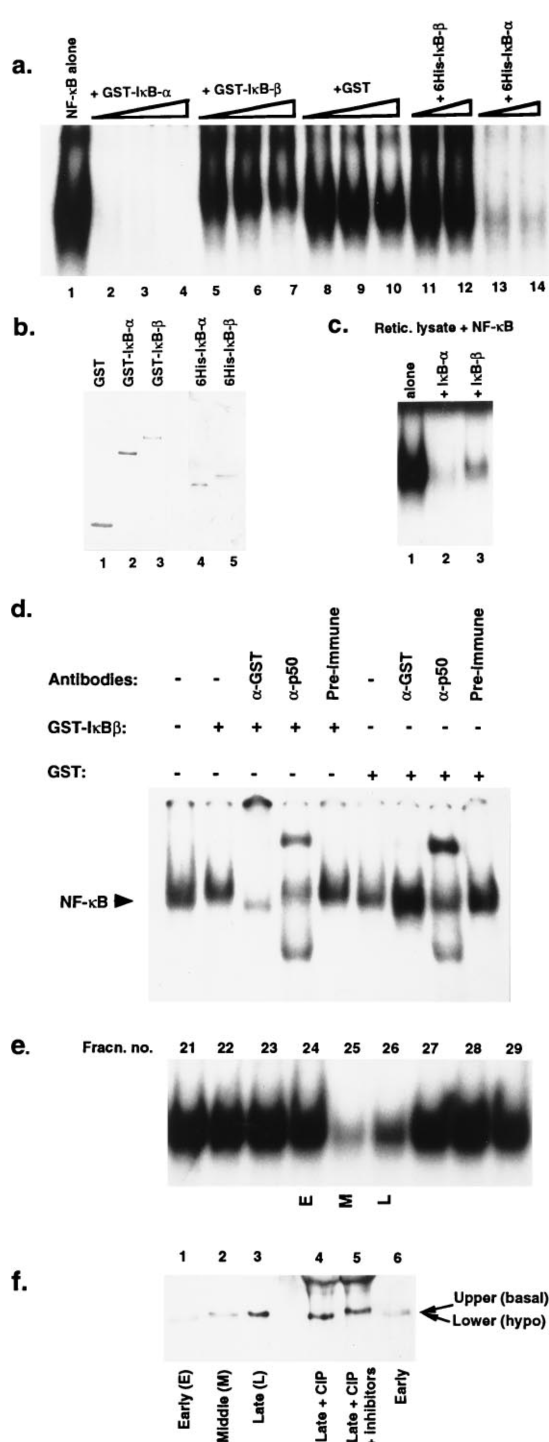

FIG. 3. Unphosphorylated IκBβ is unable to block DNA binding by NF-κB. (a) The indicated proteins were used in an inhibition EMSA with 2 μl of NF-κB purified from rabbit lungs and a radiolabeled fragment containing the canonical immunoglobulin κ site: purified NF-κB (lane 1), NF-κB with increasing amounts of GST-IκBα (lanes 2 to 4), GST-IκBβ (lanes 5 to 7), and GST (lanes 8 to 10). The presence of IκBβ in the DNA-protein complexes in the +GST-IκBβ lanes were inferred from both the slower mobility of the complex (compare mobilities in lanes 5 to 7 with that in lane 1) as well as supershifting analysis on the complex with an antibody against GST. (b) Amounts of bacterially expressed, purified GST-IκBα (lane 1), GST-IκBβ (lane 2), GST (lane 3), His-tagged IκBα (lane 4), and IκBβ (lane 5) approximately equal to those used for panel a were analyzed by Coomassie blue staining of an SDS-polyacrylamide gel. (c) Rabbit reticulocyte (Retic.) lysates contain an endogenous NF-κB that is primarily a p50-p65 heterodimer. Addition of purified rabbit NF-κB increases the signal, facilitating the subsequent assay for IκB activity. The coupled transcription-translation lysates (TNT; Promega) were supplemented with purified NF-κB for the assays. For assaying the activity of IκBα and IκBβ, the cDNAs encoding these proteins were translated in the lysates before analysis by standard EMSA using an immunoglobulin κ probe; 2 μl of the lysate was used for assay in each lane. (d) Equal amounts of purified NF-κB from rabbit lungs were incubated with GST-IκBβ or GST at room temperature for 5 min. The reaction mixtures were then further incubated with either anti-GST antibody (α-GST), anti-p50 antibody (αp50), or a preimmune serum as a control for 1 h at 4°C, and the samples were analyzed by EMSA. (e) Partially purified IκBβ proteins from COS cells were fractionated on an FPLC Mono Q column. IκBβ proteins eluting from the FPLC Mono Q column were analyzed for activity by EMSA and designated early, middle, and late fractions. Four microliters of each fraction was analyzed by immunoblotting with the IκBβ antibody. (f) Four microliters of the late fraction (fraction 26) was treated with calf intestine alkaline phosphatase (CIP) (as described for Fig. 2b) with or without phosphatase inhibitors (10 mM β-glycerophosphate and 10 mM sodium fluoride). Lane 4, treated with CIP; lane 5, treated with CIP and phosphatase inhibitors; lane 6, early fraction (fraction 24) (untreated). hypo, hypophosphorylated form.

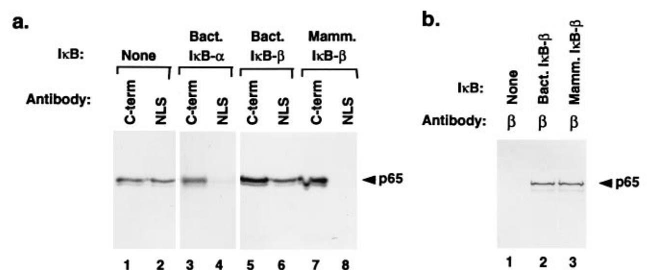

FIG. 4. Unphosphorylated IκBβ does not mask the NLS on NF-κB p65. (a) Mouse NF-κB p65 cloned in a pcDNA3 vector was translated in vitro in rabbit reticulocyte lysates. Equal amounts of the ^{35}S-labeled p65 protein were mixed with nothing (lanes 1 and 2), purified GST-IκBα (lane 3 and 4), purified GST-IκBβ (lane 5 and 6), or overexpressed, basally phosphorylated IκBβ purified from COS cells (lane 7 and 8). Following a 5-min incubation, the p65 C-terminal (C-term) peptide polyclonal antibody and p65 NLS monoclonal antibody were added as indicated. The antibody bound to p65 was precipitated with either protein A-Sepharose (C-terminal peptide antibody) or protein G-Sepharose (NLS antibody). The immunoprecipitates were fractionated by SDS-PAGE and analyzed by fluorography. Bact., bacterial; Mamm., mammalian. (b) Experiment similar to that shown in panel a except that the antibody used for immunoprecipitation was directed against IκBβ. Lanes: 1, no IκBβ; 2, GST-IκBβ; lane 3, IκBβ purified from overexpressing COS cells.

the GST portion, we produced histidine-tagged IκBα and IκBβ proteins in bacteria (Fig. 3b, lanes 4 and 5) and obtained similar results (Fig. 3a, lanes 11 to 14). In contrast, both IκBα and IκBβ translated in rabbit reticulocyte lysates were able to block NF-κB DNA binding activity (Fig. 3c, lanes 2 and 3). Therefore, although unphosphorylated IκBβ can interact with NF-κB, it cannot block DNA binding.

We were also concerned that the differences between phosphorylated and unphosphorylated IκBβ might somehow be due to expression of proteins in bacteria. To address this concern, we overexpressed IκBβ in COS cells and then purified the protein through multiple column chromatography steps. Upon fractionation on an FPLC Mono Q anion-exchange column, IκBβ protein eluted in three fractions (Fig. 3d). The protein in fraction 24, which eluted earliest, migrated faster on SDS-PAGE (Fig. 3e, lane 1), while the proteins eluting later, in fractions 25 and 26, were of slower mobility (lanes 2 and 3). This pattern is expected if the protein eluting later represents the basally phosphorylated form, which would be retarded more on an anion-exchange column. In agreement with the results presented earlier, the faster-migrating form of IκBβ, fraction 24 (unphosphorylated), was unable to block DNA binding (Fig. 3d). Treatment of the later-eluting, slower-mobility protein in fraction 26 with phosphatase altered its mobility (Fig. 3e, lane 4), but mobility was not changed in the presence of phosphatase inhibitors (lane 5), strongly suggesting that the difference between the two forms was their phosphorylation status.

Unphosphorylated IκBβ is unable to mask the NLS on NF-κB p65. The existence of an unphosphorylated IκBβ–NF-κB complex that is capable of binding to DNA suggests that this complex might enter the nucleus. However, for NF-κB bound to unphosphorylated IκBβ to enter the nucleus, the NLS on NF-κB must be exposed since it is known that NLS-mediated transport is initiated by interactions of the NLS with cytosolic NLS receptor proteins (1). It has also been established by a number of groups that binding of IκBα to NF-κB masks the NLS on NF-κB (5, 27). To test whether the binding of unphosphorylated IκBβ to NF-κB leaves the NLS on NF-κB exposed, we used an antibody directed against the NLS of p65 as a probe. It has been previously established that this antibody fails to react with the p65 NLS in a p65-IκBα complex (27).

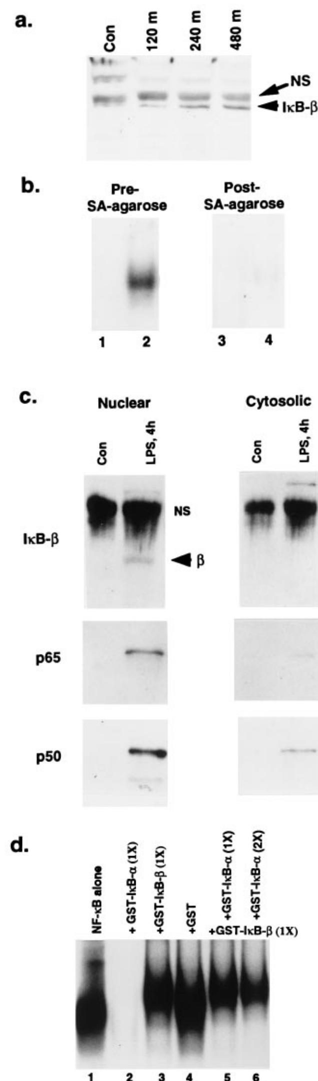

FIG. 5. IκBβ enters the nucleus in LPS-stimulated cells and can be detected in the κB-specific DNA-protein complexes. (a) Approximately 10^7 70Z/3 cells were either untreated (control [Con]) or treated with LPS (10 μg/ml) for 2, 4, and 8 h. The cytosolic and nuclear extracts were prepared, and the nuclear extract was dialyzed for 4 h against a mixture containing 100 mM NaCl, 25 mM Tris (pH 7.5), 1 mM dithiothreitol, 0.5 mM EDTA, and a cocktail of protease inhibitors. Then 30-μg aliquots of the nuclear extracts were fractionated by SDS-PAGE, and the proteins were transferred to a membrane and immunoblotted with the IκBβ antibody. NS indicates a nonspecific band that cross-reacts with the IκBβ antibody in nuclear fractions. Subsequent immunoblotting with an IκBα antibody indicates that IκBα is not detected in the nucleus (not shown). (b) Nuclear extracts from unstimulated and LPS-stimulated cells (20) were incubated with the biotinylated and radiolabeled oligonucleotide that contained the κB binding site. Precipitation of the biotinylated oligonucleotide was carried out with streptavidin-agarose (SA; lanes 3 and 4), which efficiently precipitated the NF-κB complex. The DNA protein complexes were then analyzed by EMSA and detected by autoradiography. (c) The streptavidin-agaraose precipitates from the nuclear and cytosolic extracts of unstimulated (lanes 1 and 3) and LPS-stimulated (lanes 2 and 4) cells were analyzed on equivalent SDS-polyacrylamide gels and immunoblotted with the indicated antibodies. (d) Purified NF-κB from rabbit lungs (2 μl) was first incubated with GST-IκBβ for 5 min at room temperature. The amount of IκBβ used was sufficient to cause all of the NF-κB complex to upshift (lane 3), indicating that IκBβ was in a complex with NF-κB bound to DNA. The same amount of GST-IκBα completely blocks DNA binding by NF-κB (lane 2). IκBα protein was added to the preformed GST–IκBβ–NF-κB complex up to a twofold excess and further incubated for 10 min (lanes 5 and 6).

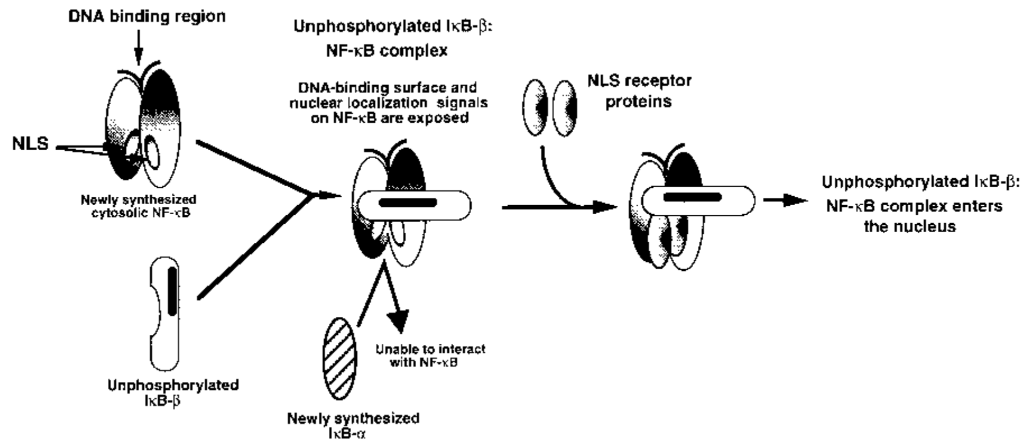

FIG. 6. Model for the regulation of NF-κB activity by IκBβ. When IκBβ is degraded, e.g., with LPS or IL-1, NF-κB activation is persistent, even though newly synthesized IκBα accumulates to levels in unstimulated cells. This is because the newly synthesized IκBβ which begins to appear (>60 min) is an unphosphorylated protein that binds to a portion of newly made NF-κB and sequesters it from IκBα. The unphosphorylated IκBβ, however, fails to mask the NLS and DNA binding domain on NF-κB, resulting in the nuclear uptake of the unphosphorylated IκBβ–NF-κB complex. This IκBβ-bound NF-κB can bind to DNA; however, it appears that either during nuclear transport or upon binding to DNA, the majority of the IκBβ is degraded.

The p65 NLS-specific antibody was compared with an antibody generated against a C-terminal peptide of p65 (Biomol). In vitro-translated p65 was incubated with an excess of purified GST-IκBα, GST-IκBβ, and basally phosphorylated IκBβ obtained from expression in COS cells (Fig. 3e and f). Immunoprecipitations were then carried out with the p65 C-terminal peptide and NLS antibodies. As shown in Fig. 4a, the p65 NLS antibody was able to efficiently immunoprecipitate p65 only when bound to unphosphorylated GST-IκBβ (lane 6). In contrast the p65 C-terminal antibody was able to immunoprecpitate p65 in all instances (lanes 3, 5, and 7). We also verified that immunoprecipitation with the IκBβ antibody brought down equal amounts of p65 bound to GST-IκBβ and basally phosphorylated IκBβ (Fig. 4b). Therefore, in contrast to IκBα–NF-κB or basally phosphorylated IκBβ–NF-κB complexes, the NLS on NF-κB bound to unphosphorylated IκBβ is exposed. This exposed NLS could bind to NLS receptor proteins promoting transport to the nucleus, and such a coupled process would therefore prevent newly synthesized IκBα from inhibiting all of the newly made NF-κB in LPS-stimulated cells.

Unphosphorylated IκBβ enters the nucleus upon prolonged stimulation. Immunoblot analysis of nuclear extracts was then performed to determine whether IκBβ could be detected in the nucleus. Such analysis revealed that the faster-migrating form of IκBβ (determined by comparing its mobility with that of IκBβ from uninduced cytosol and the 46-kDa marker [not shown]) enters the nuclei of cells stimulated with LPS (Fig. 5a). In contrast, IκBα cannot be detected in the nuclei of LPS-stimulated cells (not shown). However, the very low amount of IκBβ detected in the nucleus raises the distinct possibility that the majority of the unphosphorylated IκBβ is degraded either during nuclear transport or upon entering the nucleus. An alternative possibility is that binding to DNA by the complex of unphosphorylated IκBβ and NF-κB leads to a displacement of the IκBβ protein and its subsequent degradation. To determine whether the IκBβ detected in the nucleus is a component of the DNA-protein complex, a biotinylated κB DNA probe was synthesized and used to precipitate κB-specific complexes from LPS-stimulated cells (Fig. 5b; compare lane 4 with lane 2). The precipitated proteins were fractionated by SDS-PAGE and then subjected to immunoblotting, and the results indicated that the nuclear DNA-protein complexes from LPS-stimulated cells contained IκBβ, p65, and p50 (Fig. 5c). The lower amount of IκBβ detected in the nuclear complexes is probably a reflection of the continuing degradation of the IκBβ protein.

It has been suggested that upon activation of NF-κB, one of the mechanisms employed by newly synthesized IκBα to attenuate nuclear NF-κB is to enter the nucleus and displace NF-κB bound to DNA. To determine whether IκBα could displace the IκBβ–NF-κB complex from DNA, the following experiment was carried out. Purified NF-κB (p50-p65 complex) from rabbit lungs was incubated with GST-IκBβ to form a GST–IκBβ–NF-κB complex (Fig. 5d, lane 3). Then GST-IκBα was added to the reaction to determine whether IκBα could displace IκBβ (lanes 5 and 6). The results indicate that the complex of unphosphorylated IκBβ and NF-κB is resistant to levels of IκBα that are at least twofold greater than that required to completely block DNA binding (lane 2). Therefore, this result suggests that unphosphorylated IκBβ and NF-κB form a stable complex with DNA.

DISCUSSION

Treatment of cells with inducers such as phorbol myristate acetate leads to the phosphorylation and degradation of IκBα and the concomitant uptake of NF-κB into the nucleus. Nuclear NF-κB then causes the up-regulation of transcription of the IκBα gene, and translation of the newly transcribed IκBα mRNA results in the rapid replenishment of the depleted pool of IκBα protein (4, 8, 9, 13, 21–23). This autoregulatory loop ensures that NF-κB is activated only transiently. In contrast, persistent induction of NF-κB activity in response to certain inducers results in a sustained lowering of IκBβ protein levels (25). This observation led us to propose that persistent activation of NF-κB was regulated through the lowering of IκBβ protein levels, but the mechanism responsible for the maintaining the lowered levels of IκBβ remained to be elucidated. It was also unclear how persistently active NF-κB escaped inhibition by newly made IκBα protein.

The results presented in this report begin to provide answers to these questions. Our studies indicate that persistent, long-term induction of NF-κB activity with LPS is accompanied by the generation of an unphosphorylated form of newly synthe-

sized IκBβ. Upon stimulation, basally phosphorylated IκBβ, along with IκBα, is initially degraded. This degradation is probably the result of phosphorylation on serines 19 and 23 in IκBβ, in a manner similar to the phosphorylation of serines 32 and 36 in IκBα (11, 18). Subsequently, newly synthesized and unphosphorylated IκBβ begins to accumulate. The unphosphorylated IκBβ forms complexes with NF-κB, thus shielding those complexes from IκBα (Fig. 6). The manner in which unphosphorylated IκBβ interacts with NF-κB is different from that of basally phosphorylated IκBβ, because unphosphorylated IκBβ fails to mask the NLS and the DNA binding domain on NF-κB, and the complex of unphosphorylated IκBβ with NF-κB can thus be imported into the nucleus (Fig. 6). Therefore, the newly synthesized, unphosphorylated IκBβ plays an active, chaperone-like role in transporting a portion of newly made NF-κB to the nucleus.

The importing of the entire complex of unphosphorylated IκBβ with NF-κB into the nucleus is similar to the proposed model for p52–Bcl-3 complexes (6). Therefore the continued, persistent activation of NF-κB seen upon LPS stimulation would be due to the newly synthesized, unphosphorylated IκBβ removing a portion of the NF-κB from the inhibitory activity of IκBα, by allowing those complexes to translocate to the nucleus. However, the very low levels of unphosphorylated IκBβ that can be detected in the nuclei of stimulated cells suggest that there might be an accompanying degradation of the unphosphorylated IκBβ in the nucleus. It is possible that the binding of the unphosphorylated IκBβ–NF-κB complex to target sites on DNA results in a conformational change that causes IκBβ to be further displaced and degraded. Treatment with different proteasome inhibitors, however, fails to influence the level of IκBβ in the nucleus, suggesting that its degradation in the nucleus may occur by a pathway that does not involve proteasomes (data not shown).

An unexpected finding in this study is the extent of the conformational change that is induced by dephosphorylation of IκBβ. From the structure of NF-κB p50 and mutational analysis (7, 12, 19), it appears likely that the basally phosphorylated IκBβ masks both the pocket where the DNA sits and the NLS. Therefore, allowing the unphosphorylated IκBβ–NF-κB complex to bind to DNA would require a significant change in the manner in which the two proteins interact. This altered conformation of IκBβ bound to NF-κB also exposes the NLSs on the NF-κB subunits. We have been unable to determine whether LPS stimulation induces a phosphatase or inhibits the kinase responsible for basally phosphorylating IκBβ. Phosphatases have recently gained attention as possible regulators of transcription factors. The mammalian phosphatase calcineurin has been shown to be involved in the dephosphorylation and activation of the transcription factor NFAT, a distant relative of the Rel family (20). In this case, however, inhibitors of calcineurin had no effect on IκBβ degradation or resynthesis (data not shown). The elucidation of the mechanism responsible for generating unphosphorylated IκBβ therefore remains a challenge for the future.

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