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Regulation of IκBβ in WEHI 231 Mature B Cells

RODERICK J. PHILLIPS AND SANKAR GHOSH*

Section of Immunobiology and Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06520

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Constitutive activation of NF-κB in WEHI 231 early mature B cells resembles the persistent activation of NF-κB that is observed upon prolonged stimulation of other cells. In both cases, NF-κB DNA binding complexes are found in the nucleus, despite the abundance of cytosolic IκBα. Recently, we have shown that prolonged activation of 70Z/3 cells with lipopolysaccharide results in the degradation of IκBβ, followed by its subsequent resynthesis as a hypophosphorylated protein. This protein was shown to facilitate transport of a portion of NF-κB to the nucleus in a manner that protects it from cytosolic IκBα. We now demonstrate that the most abundant form of IκBβ in WEHI 231 cells is a hypophosphorylated protein. This hypophosphorylated IκBβ is found in a stable complex with NF-κB in the cytosol and is also detected in NF-κB DNA binding complexes in the nucleus. It is likely that hypophosphorylated IκBβ in WEHI 231 cells also protects NF-κB from IκBα, thus leading to the continuous nuclear import of this transcription factor.

The transcription factor NF-κB was originally characterized as a heterodimer composed of p50 and p65 subunits (13, 17, 27, 31). Subsequently, these proteins were found to belong to the Rel family of proteins, whose members include p52, RelB, and c-Rel (4, 26, 32, 33). These proteins share a Rel homology domain which contains motifs for homo- and heterodimerization, DNA binding, and interaction with another family of proteins termed IκBs (40). The function of the IκB proteins is to retain NF-κB in the cytosol by masking the nuclear localizing signals on Rel proteins (3). Treatment of cells with inducers such as lipopolysaccharide (LPS), interleukin 1 (IL-1), or tumor necrosis factor results in the degradation of IκB proteins and the subsequent release and nuclear translocation of NF-κB (2, 18, 40). Two major signal-responsive isoforms of IκB, IκBα and IκBβ, have been characterized and cloned (8, 15, 39). Although these IκBs are associated with similar complexes of Rel proteins, they appear to regulate NF-κB activity through distinct mechanisms. Inducers that cause the degradation of only IκBα result in the transient induction of NF-κB activity, whereas inducers that cause the degradation of both IκBα and IκBβ lead to the persistent activation of NF-κB activity (39). We have recently determined the mechanism by which persistently active NF-κB in cells stimulated with inducers such as LPS is able to avoid inhibition by newly synthesized IκBα (37). We have demonstrated that once the initial pool of IκBβ is degraded, newly synthesized IκBβ accumulates in a hypophosphorylated form. This hypophosphorylated IκBβ binds to NF-κB in an altered conformation that leaves the nuclear localizing signal on NF-κB exposed. Therefore, the portion of NF-κB that is bound to IκBβ is protected from newly synthesized IκBα in the cytosol yet is able to enter the nuclear import pathway. Hence hypophosphorylated IκBβ acts as a chaperone to ensure the continuous expression of nuclear NF-κB even in the presence of IκBα (37).

Although NF-κB is present in an inactive cytosolic form in most cells, it is constitutively active in certain cell types such as mature B cells (11, 36). In B cells, this constitutively active NF-κB is believed to be critical for the control of immunoglobulin light chain κ gene expression (1, 20, 35), consequently, determining the mechanism by which NF-κB expression itself is regulated in these cells has been an important area for investigation. Initial studies of the active NF-κB complexes in mature B-cell lines, e.g., WEHI 231 cells, revealed that they are predominantly composed of p50 and c-Rel (21, 24, 29). This contrasts with the situation in pre-B cells, where the p50 and p65 subunits are normally activated first (21, 24, 29). However, upon long-term stimulation of pre-B-cell lines with LPS, the composition of the Rel complexes changes to p50–c-Rel (21, 24, 25). This switch in Rel protein usage can be explained by the fact that the promoters for NF-κB p105 (p50) and c-Rel are themselves regulated by NF-κB (14, 38). Therefore, although the initial NF-κB complex is mainly p50-p65, after a period of time the increased amounts of p50 and c-Rel proteins result in a gradual shift of the complex to p50–c-Rel. Interestingly, along with p50 and c-Rel, the levels of IκBα mRNA also increase dramatically in cells containing active NF-κB because of the presence of three NF-κB binding sites in the IκBα promoter (6, 9, 19). Despite the increased synthesis of IκBα in B cells, such as WEHI 231 cells, it was suggested that the basis for the constitutive nuclear NF-κB in WEHI 231 cells was the increased rate of degradation of IκBα due to the phosphorylation of IκBα in response to a developmental signal.

To better understand the basis of enhanced IκBα degradation in WEHI 231 cells, we have further examined the mechanism responsible for IκBα degradation in WEHI 231 cells. As reported in this paper, neither inhibitors of the pathways leading to the phosphorylation of IκBα nor inhibitors of the proteasome responsible for degrading phosphorylated IκBα affect the level of constitutive NF-κB activity in WEHI 231 cells. It therefore appears unlikely that the degradation of IκBα in WEHI cells is due to a signal-induced, proteasome-dependent process. Instead, our results suggest that the enhanced level of IκBα degradation in these cells is most likely due to accelerated degradation of excess IκBα that is not complexed with NF-κB. To explore other mechanisms that may help explain the constitutive activation of NF-κB in these cells, we examined the role of IκBβ, which is responsible for persistent activation of NF-κB in other cells, since earlier studies did not include this IκB isoform. Our results show that a hypophosphorylated form of IκBβ which resembles the form observed in

* Corresponding author. Phone: (203) 737-4419. Fax: (203) 737-1764. E-mail: sankar.ghosh@yale.edu.

persistently activated cells can also be detected in the cytosol and nucleus of WEHI 231 cells. We therefore propose that a hypophosphorylated form of IκBβ in WEHI 231 cells acts like a chaperone to shield NF-κB from cytosolic IκBα and to continually transport a portion of the NF-κB transcription factor to the nucleus.

MATERIALS AND METHODS

Immunoblot analysis. Immunoblot analysis was performed on 30 μg of either cytosolic or nuclear extracts. Following fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane at either 25 V overnight at room temperature or 100 V for 1 h at 4°C. The membranes were then incubated with the primary antibody (IκBβ, IκBα, c-Rel, or p65 at a 1:1,000 dilution in BLOTTO plus 0.01% sodium azide) for either 4 h at room temperature or overnight at 4°C. Subsequently, the blots were washed in Tween–Tris-buffered saline and incubated with a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody for 30 min at room temperature. The immunoreactive proteins were visualized by enhanced chemiluminescence (ECL).

Immunoprecipitation assays. Approximately 2×10^7 cells per ml were incubated in medium lacking Met and Cys plus 5% dialyzed fetal calf serum (FCS) with 100 μCi of Tran^{35}S-Label per ml at 37°C for 2 h. Approximately 5×10^6 cells were used for each point of the assay. After labeling, the cells were centrifuged and lysed in cold TNT buffer (20 mM Tris [pH 7.5], 200 mM NaCl, 1% Triton X-100, and the protease inhibitors leupeptin, pepstatin, aprotinin and phenylmethylsulfonyl fluoride). The supernatant was collected, and 20 μl of protein A-Sepharose (PAS) and 2 μl of the appropriate antibody were added. This mixture was rotated overnight at 4°C, washed extensively, and analyzed by SDS-PAGE. The gel was then dried and exposed to film for 24 h.

For immunoprecipitations in which precipitated proteins were identified by immunoblot analysis, cells were not radioactively labeled but were lysed directly into the TNT buffer. Appropriate antibodies and 20 μl of PAS were added to each sample and rotated overnight at 4°C. Subsequently, the beads were washed four times in TNT buffer and boiled in 25 μl of SDS buffer, and the supernatants were analyzed by SDS-PAGE. After being transferred to a PVDF membrane, the proteins were immunoblotted with the desired antibodies and visualized by ECL.

Isolation of poly(A)$^+$ RNA and Northern analysis. Cells were grown to a density of 10^6/ml in RPMI–10% FCS–β-mercaptoethanol (0.1 mM), harvested by centrifugation and washed with phosphate-buffered saline (PBS). The cells were lysed in a solution containing 5 M guanidinium isothiocyanate, 0.05 M Tris-HCl (pH 7.5), 0.01 M Na-EDTA, and 5% β-mercaptoethanol. The DNA in the lysate was sheared by passing it through a needle, and the concentration of NaCl was adjusted to 0.5 M. An oligo(dT) tablet (Invitrogen) was added to the lysate, and the poly(A)$^+$ RNA was allowed to bind to the oligo(dT). Following washes of the oligo(dT) cellulose pellet with buffers containing progressively lower concentrations of NaCl, poly(A)$^+$ RNA was eluted with water.

Approximately 4 μg of the poly(A)$^+$ RNA was used for Northern analysis. Standard protocols for running denaturing formaldehyde agarose gels were followed. RNAs from the gel were transferred to a charged Hybond membrane with a Stratagene Posiblot apparatus. Following hybridization to the probe (labeled by random priming), the blot was washed under increasingly stringent conditions. The final wash conditions were 0.5× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 55°C.

Preparation of nuclear and cytosolic extracts. Approximately 10^7 cells were used for each point. The cells were pelleted by low-speed centrifugation, washed in PBS, and resuspended in 200 μl of buffer A (20 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and the protease inhibitors leupeptin, pepstatin, aprotinin, and phenylmethylsulfonyl fluoride). The phosphatase inhibitors sodium fluoride (5 mM) and β-glycerophosphate (10 mM) were also used when extracts were prepared for examining the phosphorylation status of IκB proteins. The cells were then put on ice and allowed to swell for 10 min before being lysed with Nonidet P-40 (NP-40) detergent. Following centrifugation, the nuclear pellet was washed once more in 100 μl of buffer A and then resuspended in 50 μl of buffer C (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, and the protease inhibitors mentioned above). The supernatant recovered from the NP-40 lysis solution was further centrifuged at $90,000 \times g$ for 30 min at 4°C to remove cellular debris; this supernatant comprised the cytosolic extract. The nuclear pellet was shaken for 15 min at 4°C and then centrifuged at $10,000 \times g$ to remove debris. Protein concentrations were then determined with the Micro BCA protein assay kit (Pierce), and the nuclear and cytosolic extracts were stored at −70°C in the presence of 10% glycerol.

Gel shift assay. Ten micrograms of nuclear extract was added to a cocktail containing the ^{32}P-labeled κB probe (from the immunoglobulin κ gene intronic enhancer), dI-dC (competitor DNA), GTP, bovine serum albumin, and binding buffer. Following incubation at room temperature for 15 min, the samples were electrophoresed under standard conditions and dried, and the gels were exposed to film for approximately 24 h (12).

Detection of DNA-binding proteins with a biotinylated κB probe. Approximately 5×10^7 cells were used for each point. Nuclear and cytosolic extracts were prepared from WEHI 231 cells by the protocol described above. The extracts were precleared by mixing with PAS and were washed with PBS, and then they were precleared again with streptavidin-agarose. The precleared extracts were then incubated with the biotinylated κB probe in the presence of excess poly(dI-dC), which acts as competitor DNA. Streptavidin-agarose beads were then added, and the mixture was rotated at 4°C for 1 h. After this time the beads were centrifuged and washed 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. The beads were resuspended in 25 μl of SDS sample buffer, boiled, and centrifuged, and the supernatant was analyzed by SDS-PAGE. The proteins were transferred to PVDF membranes, incubated with the appropriate antibodies, and visualized by ECL.

Antibodies and reagents. The IκBα antibody was generously provided by Nancy Rice (NCI, Frederick, Md.), while the proteasome inhibitor Z-LLF-CHO was kindly provided by F. Mercurio (Signal Pharmaceuticals, San Diego, Calif.). The c-Rel and p65 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.), whereas the IκBβ polyclonal antiserum was prepared on site. Both PAS and dI-dC were purchased from Pharmacia Biotech (Uppsala, Sweden). Tran^{35}S-Label was obtained from ICN Pharmaceuticals (Costa Mesa, Calif.), the Micro BCA protein assay kit was from Pierce (Rockford, Ill.), PVDF (Immobilon P) membranes were from Millipore (Bedford, Mass.), the ECL kit was from Amersham Life Science (Arlington Heights, Ill.), calpain inhibitor I was from Calbiochem (La Jolla, Calif.), lactacystin was from Biomol, and okadaic acid was from Gibco BRL (Gaithersburg, Md.).

RESULTS

The constitutively active NF-κB in WEHI 231 cells is not affected by PDTC, calpain inhibitor I, or lactacystin. The treatment of cells with inducers of NF-κB such as LPS or IL-1 leads to the phosphorylation and subsequent degradation of IκBα and IκBβ. It is known that antioxidants such as pyrrolidone dithiocarbamate (PDTC) interfere with the signaling pathways leading to NF-κB and block the inducible phosphorylation of IκB proteins (34). Once phosphorylated, IκB proteins are rapidly ubiquitinated and degraded by the multicatalytic proteasome, and inhibitors such as calpain inhibitor I and lactacystin block the degradation of the phosphorylated, ubiquitinated IκB proteins (2, 40). To test whether the constitutive NF-κB activity seen in the nucleus of WEHI 231 cells is the result of continuous signal-dependent degradation of IκB proteins, we treated WEHI 231 cells with either PDTC, calpain inhibitor I, or lactacystin. In all cases, there was no effect on either the level of nuclear NF-κB DNA binding activity or the amount of IκBα and IκBβ in these cells (Fig. 1). However, the same concentration of these inhibitors was able to block the activation of NF-κB in LPS-stimulated pre-B cells (data not shown). This result strongly suggests, therefore, that the constitutive activation of NF-κB in WEHI 231 cells is unlikely to be the result of a continuous signal and proteasome-dependent degradation of IκB proteins.

The maintenance of the constitutively active nuclear NF-κB in mature B cells is not due to the establishment of an autoregulatory feedback loop. The activation of NF-κB in most cells is accompanied by the transcriptional up-regulation of NF-κB-regulated genes, including genes encoding p50 and c-Rel (21, 24). The composition of the nuclear complexes in WEHI cells, namely p50–c-Rel heterodimers, is believed to be the result of NF-κB-directed elevation in the levels of p50 and c-Rel in these cells. We therefore wanted to determine whether the maintenance of nuclear NF-κB in mature B cells was due to a feedback mechanism that led to the perpetuation of the nuclear NF-κB activity through increased synthesis of p50 and c-Rel. We began by treating 70Z/3 cells for extended periods with LPS, which leads to the persistent activation of NF-κB in these cells (39) (Fig. 2a). An examination of nuclear extracts with immunoblots clearly indicated the presence of c-Rel in the nucleus (Fig. 2a, lane 2), indicating that the NF-κB complexes in these cells were switching from p50-p65 to p50–c-Rel. However, removing LPS from the medium led to a loss of nuclear c-Rel and a significant reduction in NF-κB activity,

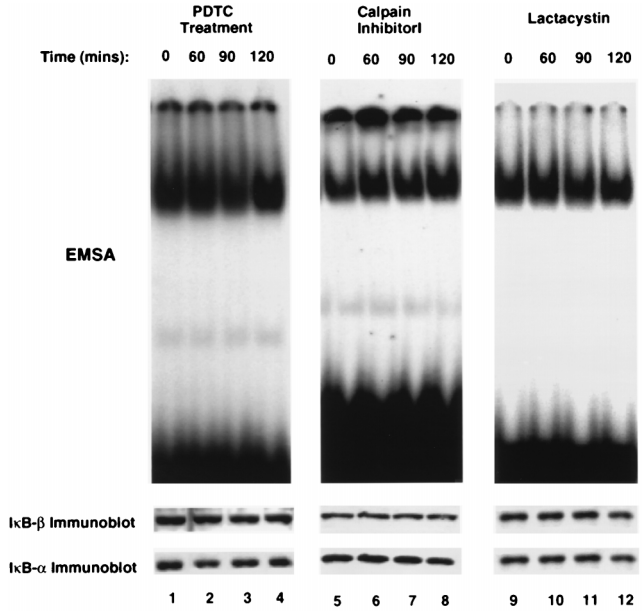

FIG. 1. PDTC, calpain inhibitor I, and lactacystin do not affect constitutively active NF-κB in WEHI 231 cells. WEHI 231 cells were treated with either PDTC (100 μM), calpain inhibitor I (250 μM), or lactacystin (10 μM) for the indicated periods of time. Nuclear and cytoplasmic extracts were then prepared. Nuclear extracts were used for electrophoretic mobility shift assay (EMSA) (upper panels), while the cytosolic extracts were used for immunoblots with IκBα and IκBβ antibodies (lower panels).

indicating that maintenance of the nuclear signal requires a continuous signal (Fig. 2a, lanes 3 to 6). We extended our analysis by treating WEHI 231 cells with tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK), an inhibitor of chymotrypsin which also inhibits inducible NF-κB activity (16). Treatment of WEHI 231 cells with 50 μM TPCK for 1 h completely inhibited NF-κB activity and therefore allowed us to determine the contribution of nuclear NF-κB to the persistence of the constitutive activation of NF-κB in these cells. Treatment with TPCK was accompanied by a loss of c-Rel from the nucleus (Fig. 2b), and this inhibitory effect was not dependent on the accumulation of either IκBα or IκBβ (Fig. 2b, lower panels). Furthermore, once TPCK was removed from the media, nuclear NF-κB reappeared within 30 min (Fig. 2c). Taken together, these results suggest that the constitutive activation of NF-κB in WEHI 231 cells requires an active mechanism, although the establishment of the feedback loop through increased synthesis of p50 and c-Rel is clearly necessary to ensure that the nuclear complex is a p50–c-Rel heterodimer.

Increased levels of IκBα mRNA in WEHI 231 cells are not accompanied by increased levels of IκBα protein. The level of IκBα mRNA detected in WEHI 231 cells was approximately 50- to 100-fold greater than the level in pre-B cells (Fig. 3a). However, immunoblotting of extracts from these cells indicated no significant difference in the levels of IκBα protein between pre-B cells and mature B cells (Fig. 3b). Therefore, the vast majority of the new IκBα synthesized in WEHI cells appears to be degraded, most likely because it is in excess of the available NF-κB and free IκBα is unstable. Pulse-chase analysis has indicated that IκB-α is more rapidly turned over in WEHI 231 cells than in 70Z/3 pre-B cells (23, 30), although

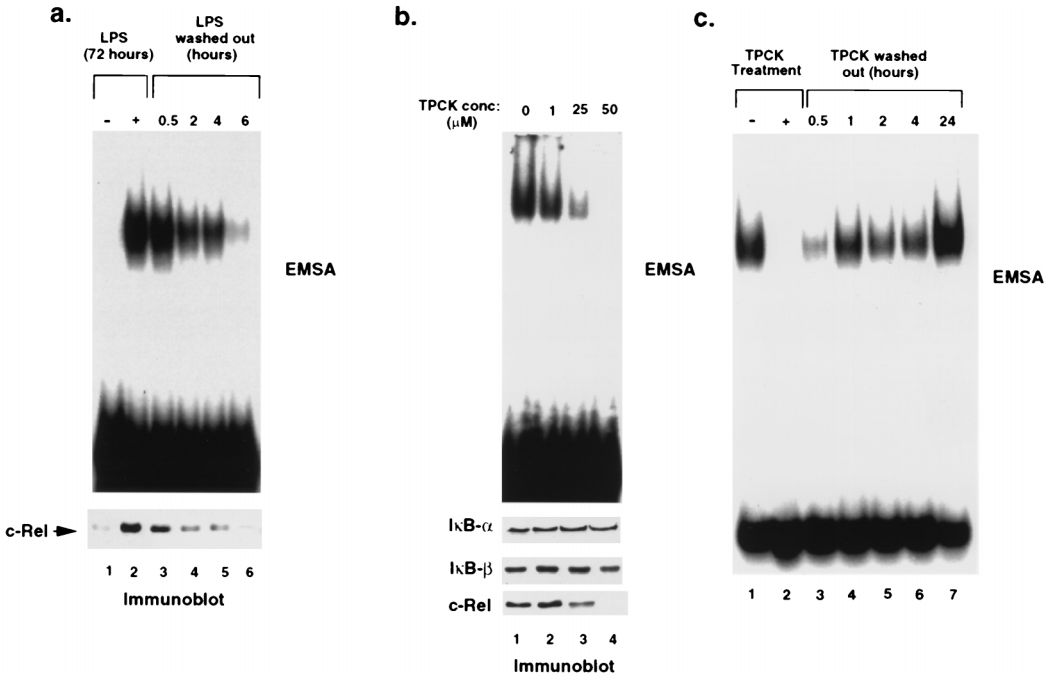

FIG. 2. Persistent activation is necessary to maintain constitutive activation of NF-κB in pre-B cells but not in mature B cells. (a) 70Z/3 cells were either unstimulated (lane 1) or stimulated for 72 h with LPS (10 μg/ml) (lane 2). Subsequently, the cells were washed extensively to remove LPS and then further incubated at 37°C. At the times indicated, aliquots of the cells were removed and nuclear extracts were prepared for electrophoretic mobility shift assay (EMSA) (upper panel, lanes 3 to 6) or immunoblot analysis (lower panel, lanes 3 to 6). (b) WEHI 231 cells were either untreated (lane 1) or treated with the indicated concentrations of the protease inhibitor TPCK for 1 h (lanes 2 to 4). Nuclear extracts were then prepared and examined by EMSA (upper panel) and immunoblotted with c-Rel antibodies, whereas cytoplasmic extracts were immunoblotted with IκBα and IκBβ antibodies (lower panel). (c) WEHI 231 cells were either untreated (lane 1) or treated with TPCK (50 μM for 1 h) (lane 2). Subsequently, the cells were washed extensively to remove the TPCK and incubated further. The cells were harvested at the indicated times, and nuclear extracts were prepared and used for gel shift analysis (lanes 3 to 7).

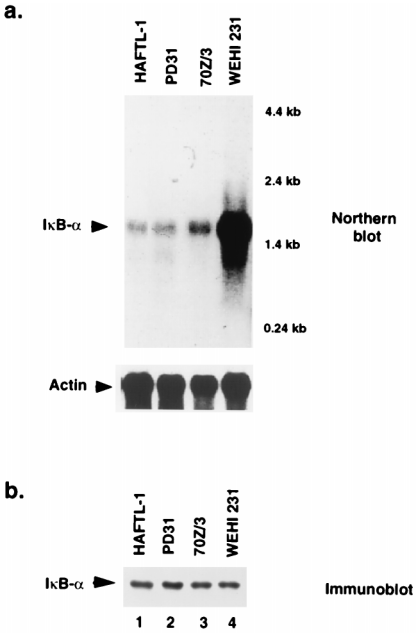

FIG. 3. IκBα mRNA, but not protein levels, are greatly up-regulated in WEHI 231 cells. (a) Northern analysis of poly(A)$^+$ RNA prepared from the B-cell lines HAFTL-1 (pro-B cells), PD31 (pre-B cells), 70Z/3 (pre-B cells), and WEHI 231 (early mature B cells). Four micrograms of RNA from each cell type was analyzed. The blots were probed with IκBα, and the hybridized probe was detected by fluorography. The amount of RNA in each lane was determined by hybridizing to actin. (b) Cytoplasmic extracts were prepared from a panel of B-cell lines (HAFTL-1, PD31 and 70Z/3, and WEHI 231 cells) at different stages of development, as described above. These extracts were then examined for IκBα protein levels by using immunoblotting techniques.

such analysis does not reveal whether the faster turnover is confined to the free IκBα pool or whether it includes the portion of IκBα that is bound to NF-κB in the cytosol. If the IκBα bound to NF-κB in WEHI 231 cells is rapidly degraded in response to a developmental signal, it is reasonable to assume that treatment of WEHI cells with inducers of NF-κB activity that target cytosolic NF-κB–IκBα complexes should not further affect the rate of IκBα degradation. To test this hypothesis, we stimulated WEHI 231 cells with LPS (Fig. 4). Surprisingly, the treatment of WEHI 231 cells with LPS causes both IκBα and IκBβ to be rapidly degraded and resynthesized (apparent for IκBα) in a manner identical to that seen in pre-B cells (Fig. 4 [lower panel], lanes 2 to 5). The resynthesis of IκBα at later time points could be blocked by cycloheximide, indicating the involvement of new protein synthesis (data not shown). This suggests that the pathways responsible for signal-induced degradation of IκBs are not constitutively active in WEHI 231 cells. We next examined the effect of lactacystin, a specific proteasome inhibitor that blocks signal-induced degradation of IκBs, on LPS-induced WEHI 231 cells. Addition of lactacystin blocked the LPS-induced degradation of IκBα and IκBβ in WEHI 231 cells in a manner similar to that observed in 70Z/3 pre-B cells (Fig. 4 [lower panel], lanes 6 to 9, and data not shown). Surprisingly, there was no detectable accumulation of phosphorylated IκBα, which can be identified by its slightly slower mobility on SDS-PAGE gels (Fig. 4, lower panel). Our failure to observe the hyperphosphorylated form of IκBα could be due to the presence of a phosphatase activity in these cells (discussed later). These results, therefore, argue against the hypothesis that IκBα in WEHI 231 cells is subject to signal-induced phosphorylation and degradation. Furthermore, these results strongly suggest that the more rapid turnover of IκBα that is observed in WEHI 231 cells is not related to signal-induced, proteasome-mediated degradation but is instead due to the degradation of free, uncomplexed IκBα.

The major form of IκBβ in WEHI 231 cells is a hypophosphorylated, faster-migrating protein. We recently demonstrated that during persistent activation of 70Z/3 cells with inducers such as LPS, newly synthesized, hypophosphorylated IκBβ appears to act as a chaperone-like molecule that shields NF-κB from IκBα and facilitates its translocation to the nucleus. Although the majority of the IκBβ is degraded during the nuclear import process, we were able to demonstrate that a small portion of the nuclear NF-κB complex contains IκBβ, suggesting that the IκBβ–NF-κB complex is imported to the nucleus prior to its degradation (37). Based on these findings, we wanted to examine whether the regulation of NF-κB in WEHI 231 cells might also involve IκBβ. We began by comparing the mobility of IκBβ from WEHI 231 cells with IκBβ from pre-B cells (Fig. 5a). The immunoblot indicated that the majority of the IκBβ in WEHI 231 cells was of a faster-migrating form that corresponded to the mobility of the hypophosphorylated protein (Fig. 5a, lane 4). Furthermore, alkaline phosphatase treatment of the IκBβ from 70Z/3 pre-B cells, but not from WEHI 231 early mature B cells, gave rise to a faster-migrating form of IκB (Fig. 5b; compare lanes 1 and 2 with lanes 4 and 5). These results suggested that the IκBβ protein found in WEHI 231 cells was mostly hypophosphorylated, and it was likely, therefore, that this form of IκBβ was involved in regulating the constitutive activation of NF-κB in these cells.

The hypophosphorylated form of IκBβ in WEHI 231 cells forms a complex with NF-κB. Our previous study had indicated that hypophosphorylated, newly synthesized IκBβ binds to a portion of NF-κB in the cytosol and thus protects that

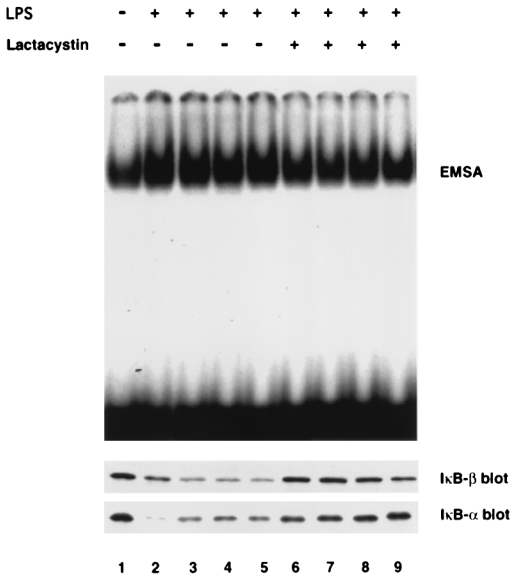

FIG. 4. LPS-induced degradation of IκBα in WEHI 231 cells is abrogated in the presence of the proteasome inhibitor lactacystin. WEHI 231 cells were either untreated (lanes 1 to 5) or pretreated with lactacystin (10 μM for 1 h) (lanes 6 to 9). Subsequently, the cells were stimulated with LPS (10 μg/ml) (lanes 2 to 9) for the following time periods: 30 min (lanes 2 and 6), 60 min (lanes 3 and 7), 90 min (lanes 4 and 8), and 120 min (lanes 5 and 9). Upon termination of the experiment, nuclear and cytoplasmic extracts were prepared. κB-binding complexes in the nuclear fraction were examined by electrophoretic mobility shift assay (EMSA), while IκBα and IκBβ protein levels in the cytoplasmic fraction were examined by immunoblot analysis.

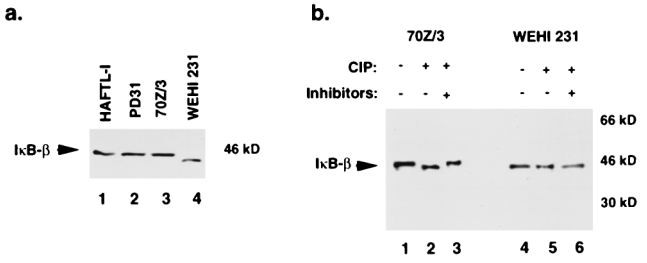

FIG. 5. IκBβ in WEHI 231 cells is hypophosphorylated. (a) Cytoplasmic extracts were prepared from a panel of B-cell lines at different stages of development (as described in the legend to Fig. 3). Thirty micrograms of each extract was fractionated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with IκBβ antibody. (b) Cytoplasmic extracts from WEHI 231 cells and 70Z/3 cells were either untreated (lanes 1 and 4) or treated with calf intestinal phosphatase (CIP) (20 U) (lanes 2 and 5) or CIP plus the phosphatase inhibitors β-glycerophosphate and sodium fluoride (10 mM each) (lanes 3 and 6). The reactions were performed at 30°C for 1 h; after this period the samples were fractionated by SDS-PAGE and immunoblotted with IκBβ antibody. The difference in mobility of IκBβ from those of 70Z/3 cells and WEHI 231 cells is very small and is apparent only when analysis with long SDS-PAGE gels (10% polyacrylamide) is made.

portion of NF-κB from IκBα (37). Therefore, we wanted to determine whether the hypophosphorylated IκBβ seen in WEHI 231 cells was also complexed with NF-κB in the cytosol. We labeled WEHI 231 and 70Z/3 cells with [^{35}S]methionine and immunoprecipitated them with antibodies against IκBβ (Fig. 6a). Under these conditions, two bands at around 66 kDa were observed in 70Z/3 cells, whereas only the upper band was observed in WEHI 231 cells (Fig. 6a; compare lanes 1 and 3). It is likely that the upper band is c-Rel, which has a molecular mass of about 69 kDa while the lower band is probably p65, which has a molecular mass of 65 kDa. Interestingly, the ^{35}S-labeled IκBβ band (around 46 kDa) in WEHI 231 cells exhibited a slightly faster mobility (Fig. 6a, lanes 1 and 3). To confirm these results, we performed additional immunoprecipitations from unlabeled cells with antibodies against IκBβ, fractionated the immunoprecipitates by SDS-PAGE, and then immunoblotted with either p65 or c-Rel antibodies (Fig. 6b and c). We observed that in 70Z/3 cells, IκBβ was bound to both p65 and c-Rel, while in WEHI 231 cells, IκBβ was mainly coimmunoprecipitated with c-Rel (Fig. 6c; compare lanes 2 and 4). These results therefore indicate that hypophosphorylated IκBβ in WEHI 231 cells exists in a complex with Rel proteins.

We have previously shown that the complex of hypophosphorylated IκBβ and NF-κB differs from the complex of basally phosphorylated IκBβ and NF-κB in another significant manner: whereas the binding of basally phosphorylated IκBα and IκBβ to NF-κB blocks the ability of NF-κB to bind DNA, the hypophosphorylated form of IκBβ is unable to block DNA binding (7, 37). Therefore, if the complex of hypophosphorylated IκBβ–NF-κB enters the nucleus, it should be able to bind to DNA. Immunoblot analysis of WEHI 231 nuclear cell extracts indicated that a small amount of IκBβ could be detected in the nucleus (Fig. 7a, lane 4). Parallel blots indicated that unlike IκBβ, no IκBα could be detected in the nuclear fractions (Fig. 7a). To determine whether this IκBβ in the nucleus is a component of the NF-κB bound to DNA, we used a biotinylated κB oligonucleotide to precipitate proteins bound specifically to the κB sequence (Fig. 7b). The precipitates were analyzed by SDS-PAGE followed by immunoblotting. The results clearly indicated the presence of p65, c-Rel, and IκBβ in these nuclear κB-specific complexes from WEHI 231 cells, but no IκBα. However, as we had seen previously with LPS-induced complexes, the amount of IκBβ was far less than that of the other proteins, suggesting that the majority of the IκBβ was being degraded, either during import into the nucleus or inside the nucleus.

DISCUSSION

The mechanism by which a portion of NF-κB remains constitutively active in mature B-cell lines has remained a mystery despite the progress made in elucidating many aspects of the regulation of NF-κB/Rel proteins. Enhanced IκBα degradation has been suggested to be responsible for this phenomenon (23, 30); however, this theory does not explain how a modest increase in the rate of IκBα degradation could overcome the significant excess of IκBα mRNA, and therefore protein, produced in these cells and still lead to the appearance of constitutively active, nuclear NF-κB. Instead, the observed increase in the rate of IκBα degradation in WEHI 231 cells might be more easily explained by postulating that the newly synthesized IκBα, which is in excess of available NF-κB, is more rapidly

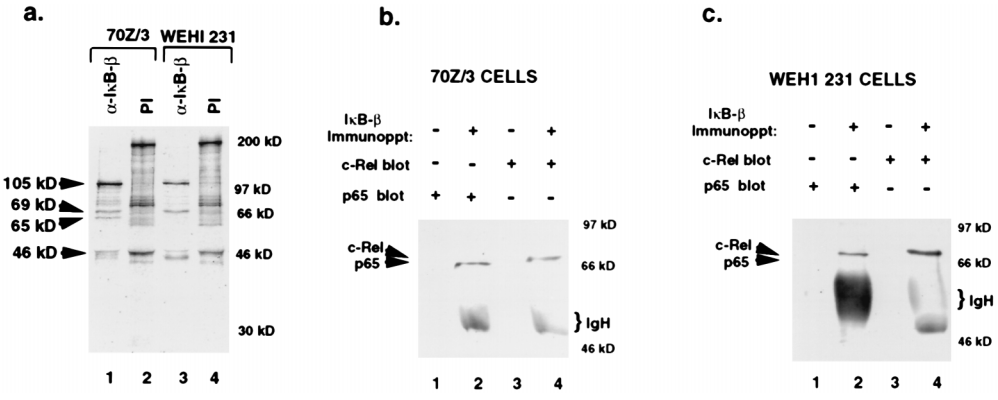

FIG. 6. IκBβ associates with both c-Rel and p65 in WEHI 231 cells. (a) 70Z/3 (lanes 1 and 2) and WEHI 231 (lanes 3 and 4) cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Immunoprecipitations were performed on total cell lysates with antiserum to IκBβ (lanes 1 and 3) and the corresponding preimmune serum (PI) (lanes 2 and 4). After being washed extensively, the samples were fractionated by SDS-PAGE and exposed to film for 24 h. Only the bands detected with the immune serum are indicated (lanes 1 and 3). (b and c) Cells (2×10^7), either 70Z/3 or WEHI 231, were lysed directly into TNT buffer. The resulting total lysate was divided into four aliquots and immunoprecipitated with (lanes 2 and 4) or without (lanes 1 and 3) IκBβ antibody. The immunoprecipitates were fractionated by SDS-PAGE and transferred to PVDF membranes. These membranes were then immunoblotted with either p65 or c-Rel antibodies as indicated. IgH, immunoglobulin H.

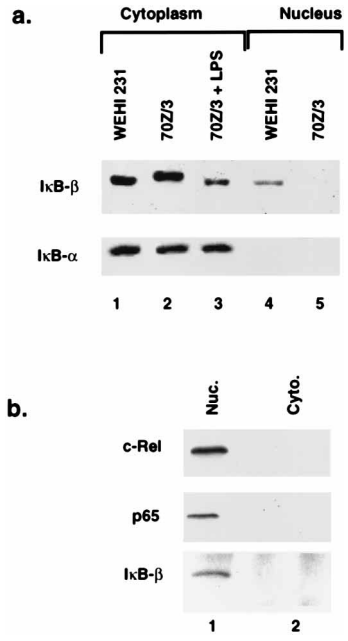

FIG. 7. Hypophosphorylated IκBβ is present in nuclear NF-κB DNA binding complexes. (a) Nuclear (lanes 4 and 5) and cytoplasmic (lanes 1 to 3) extracts of both 70Z/3 (lanes 2, 3, and 5) and WEHI 231 (lanes 1 and 4) cells were fractionated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with IκBβ antibody. (b) Nuclear and cytoplasmic extracts from WEHI 231 cells were incubated with a biotinylated oligonucleotide that contained the κB binding site. The biotinylated oligonucleotide was then precipitated with streptavidin-agarose beads, washed extensively, and fractionated by SDS-PAGE. The nuclear (lane 1) and cytoplasmic (lane 2) extracts were then immunoblotted with either c-Rel, p65, or IκBβ antibodies as indicated.

degraded (23, 30). The inadequacy of the current model in explaining the constitutive activation of NF-κB in WEHI 231 cells led us to explore alternative mechanisms that might better explain this phenomenon. Recent findings demonstrating that IκBβ regulates the persistent activation of NF-κB in 70Z/3 cells stimulated with LPS (37, 39) and in human T-cell leukemia virus type 1-transformed T cells (22) have instead raised the possibility that the constitutive activation of NF-κB in WEHI 231 cells might mimic the persistent activation of NF-κB in other cells and be regulated in a similar manner by IκBβ.

The results presented in this report provide evidence that in WEHI 231 B cells, IκBβ is found predominantly as a hypophosphorylated protein in both the nucleus and the cytosol. In the cytosol, IκBβ is detected in complexes with Rel proteins, whereas in the nucleus, IκBβ is associated with Rel complexes bound to DNA. Therefore, it appears that, like the mechanism responsible for persistent activation of NF-κB in response to inducers such as LPS, IκBβ is also responsible for ensuring that a portion of NF-κB is continually transported to the nucleus in WEHI 231 cells (37). Although the composition of NF-κB in WEHI 231 cells (i.e., the p50–c-Rel heterodimer) is due to the establishment of a feedback loop where the amount of p50 and c-Rel is elevated by the presence of nuclear NF-κB, our studies suggest that this feedback loop by itself is not sufficient to maintain nuclear NF-κB, and a continuous signal is necessary. We suggest that this continuous signal causes IκBβ to become hypophosphorylated, and this hypophosphorylated protein binds to a portion of cytosolic NF-κB and helps to transport it to the nucleus. The exact mechanism by which IκBβ is maintained in this hypophosphorylated state in WEHI 231 cells is unclear at present, but two possibilities exist. First, up-regulation or constitutive activation of an IκBβ-specific phosphatase might occur; alternatively, down-regulation or inactivation of an IκBβ-specific kinase responsible for basally phosphorylating IκBβ might take place. At present, we are unable to distinguish between these possibilities, although it seems likely that both a phosphatase and a kinase are active in WEHI 231 cells, since we have observed that in the presence of the phosphatase inhibitor okadaic acid, hypophosphorylated IκBβ becomes hyperphosphorylated (28). The ability of LPS to cause the inducible degradation of IκBα and IκBβ in WEHI 231 cells strongly suggests that an inducible IκB kinase is present and can be activated in these cells and that sites of basal phosphorylation on IκBβ are distinct from the N-terminal serine residues. It is important to note that the phosphorylation of two serine residues in the C-terminal PEST domain of IκBβ has been shown recently to affect the ability of IκBβ to inhibit DNA binding by NF-κB (7). Although these sites for basal phosphorylation can be phosphorylated by casein kinase II, it is not clear whether casein kinase II mediates the site-specific phosphorylations in the PEST region of IκBβ in vivo. However, it appears likely that a phosphatase specific for IκBβ exists, since in both the constitutive activation of NF-κB observed in WEHI 231 cells and the persistent activation of NF-κB observed in 70Z/3 cells stimulated with LPS, only IκBβ is found in a hypo- or unphosphorylated state. Thus, in order to maintain IκBβ in a hypo- or unphosphorylated state without affecting the phosphorylation status of IκBα, a phosphatase that can discriminate between IκBα and IκBβ would be required. Identifying this phosphatase would therefore be an important step in further analyzing the mechanism responsible for constitutive activation of NF-κB in WEHI 231 cells.

Although the proposed role for unphosphorylated IκBβ as a chaperone provides the best explanation for the constitutive activation of NF-κB in WEHI 231 cells at present, a number of issues remain unresolved. The small amount of IκBβ observed in the nucleus suggests that there is an ongoing degradation of IκBβ concomitant to nuclear transport or upon the binding of NF–κB–IκBβ complexes to DNA. However, this degradation of IκBβ in WEHI 231 cells is not affected by the addition of proteasome inhibitors such as calpain inhibitor I or lactacystin, suggesting that a distinct proteolytic pathway affecting IκBβ exists in these cells. Another question raised by our findings is the state of the cytosolic NF-κB–IκBβ complexes in WEHI 231 cells. We have demonstrated previously that the association of unphosphorylated IκBβ with NF-κB fails to mask the nuclear localizing signals on NF-κB, thereby allowing the complex of NF-κB and unphosphorylated IκBβ to enter the nucleus (37). However, we were able to detect significant amounts of NF-κB–IκBβ complexes in the cytosol in WEHI 231 cells. It is possible that while the unmasking of the nuclear localizing signals in the NF-κB–IκBβ complexes is an important step, there are probably additional steps that are required before the NF-κB–IκBβ complex can enter the nucleus. Alternatively, the cytosolic complexes of hypophosphorylated IκBβ–NF-κB complexes in WEHI 231 cells may contain an additional component that regulates the translocation of this complex to the nucleus. Further studies will be necessary to resolve this issue.

Signal-induced degradation of both IκBα and IκBβ represents one layer of regulation for IκB proteins, and their basal phosphorylation represents a second. The signal transduction pathways that target IκB proteins for degradation have yet to be elucidated. In particular, it is unknown whether the signaling pathway that promotes degradation of IκBα and hence mediates the transient activation of NF-κB is the same pathway that regulates degradation of IκBβ and the persistent (or

constitutive) activation of NF-κB. Recent work has demonstrated that during the initial activation of NF-κB, IκBα is phosphorylated on serine 32 and serine 36, which leads to ubiquitination on lysine 21 and 22 and the subsequent degradation of IκBα by the 26S proteasome (2, 40). The so-called IκBα kinase that mediates these specific phosphorylations on the N-terminal serine residues has yet to be cloned, although a 700-kDa multisubunit kinase has recently been partially purified and shown to specifically phosphorylate IκBα (5). The activity of this kinase was studied only on IκBα, and it remains to be determined whether this kinase is also able to phosphorylate IκBβ. It has already been shown that IκBβ probably undergoes specific signal-mediated phosphorylation similar to IκBα on two N-terminal serines at positions 19 and 23, a process that is also followed by proteasome-mediated degradation (10). However, since in some cells IκBβ responds only to a subset of activators such as LPS and IL-1 and not to others, such as phorbol myristate acetate (39), it is possible that distinct kinases are involved in the specific phosphorylation of IκBα and IκBβ. If so, this would imply that all the enzymes regulating the activity of IκBα and IκBβ are distinct and that, therefore, the pathways regulating them are compartmentalized from each other. Our finding that only the pathway affecting IκBβ seems to be active in WEHI 231 cells raises the possibility that these cells might serve as a model system for understanding the mechanism by which the differential regulation of IκBα and IκBβ activity is achieved.

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