MOLECULAR AND CELLULAR BIOLOGY, May 1995, p. 2689–2696 Vol. 15, No. 5
0270-7306/95/$04.00+0

Inducible Nuclear Expression of Newly Synthesized IκBα Negatively Regulates DNA-Binding and Transcriptional Activities of NF-κB

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Received 24 August 1994/Returned for modification 13 October 1994/Accepted 8 February 1995

The transcription factor NF-κB is exploited by many viruses, including the human immunodeficiency virus, for expression of viral genes, but its primary role appears to be in the rapid induction of cellular genes during immune and inflammatory responses. The inhibitor protein IκBα maintains NF-κB in an inactive form in the cytoplasms of unstimulated cells, but upon cell activation, IκBα is rapidly degraded, leading to nuclear translocation of free NF-κB. However, NF-κB-dependent transcription of the IκBα gene leads to rapid resynthesis of the IκBα protein and inhibition of NF-κB-dependent transcription. Here we demonstrate a new regulatory function of IκBα exerted on NF-κB in the nuclear compartment. Although normally found in the cytoplasm, IκBα, newly synthesized in response to tumor necrosis factor or interleukin 1, is transported to the nucleus. In the nucleus IκBα associates with the p50 and p65 subunits of NF-κB, inhibiting DNA binding of the transcription factor. Furthermore, nuclear expression of IκBα correlates with transcription termination of transfected NF-κB-dependent luciferase genes. Following the appearance of IκBα in the nuclei of activated cells, a dramatic reduction in the amount of nuclear p50 occurs, suggesting that NF-κB–IκBα complexes are cleared from the nucleus.

Nuclear factor κB (NF-κB) is a sequence-specific DNA-binding protein complex which regulates the expression of viral genomes, including the human immunodeficiency virus (HIV), and a wide variety of cellular genes, particularly those involved in immune and inflammatory responses (for a review, see reference 6). NF-κB is composed of two polypeptide species with molecular weights of 50,000 (p50) and 65,000 (p65) (7, 27). Cloning of the p50 (21, 29) and p65 (40, 44) subunits of the NF-κB heterodimer showed that they belong to a multigene family (the *rel* family) of proteins, all of which are implicated in transcriptionally regulated processes, such as the expression of cytokines, acute-phase response genes, and the determination of the dorsoventral polarity in early insect embryos (for a review, see reference 22).

A major component of the regulation of p50-p65 complex activity is the control of the intracellular localization of the heterodimer. Indeed, in most cell types, NF-κB is retained in an inactive form in the cytoplasm by the inhibitor protein IκBα (for a review, see reference 6). Induction of NF-κB by extracellular or intracellular stimuli has been demonstrated in a large number of cell types. Thus, it has been shown that following cell stimulation by a number of inducers, such as specific antigen recognition, tumor necrosis factor (TNF), interleukin 1 (IL-1), UV light, phorbol esters, bacterial lipids, oxygen radicals, or DNA and RNA virus infections (for a review, see reference 6), the inhibitory capacity of IκBα is lost and transcriptionally active NF-κB is translocated into the nucleus. IκBα, or MAD-3 (23), belongs to a family of proteins including IκBβ (50), Bcl-3 (41), cactus (18, 28), and the carboxy-terminal region of p105 IκBγ (11, 26, 33, 43), which are characterized by their capacity to bind one or more NF-κB/rel family members and to contain multiple ankyrin domains which are thought to adopt a common structural motif. Interaction of IκB proteins with the nuclear localization signals (NLS) of NF-κB proteins can result in the inhibition of the NF-κB proteins' DNA-binding activity (32), with the protein complex being held in the cytoplasm because of the masking of the NLS (9, 36).

At present the posttranslational events leading to inactivation of IκBα are poorly understood. The inability of in vitro-phosphorylated IκBα to bind NF-κB (20) suggests that in vivo phosphorylation of the inhibitor following cell stimulation by TNF, IL-1, poly(rI)·poly(rC) or the human T-cell leukemia virus type 1 Tax protein (8, 37, 47) may determine its dissociation from p50-p65 heterodimers. However, it has been recently reported that, in vivo, at least part of phosphorylated forms of IκBα remain associated with NF-κB upon cell activation (16, 47, 49). These findings suggest that phosphorylation of IκBα per se is not sufficient to induce nuclear translocation of NF-κB, although it could mark the molecule for the rapid proteolysis undergone upon cell activation (8, 13, 25, 37, 48). In these cases, IκBα degradation may occur even while the inhibitor is physically associated with NF-κB in the cytoplasm. Suppression of IκBα degradation in cell cultures treated by synthetic peptide-aldehydes, which efficiently block the in vitro proteolytic activity of the proteasome, indicates that this entity could be involved in the inducible degradation of the inhibitor observed in vivo (42, 49). After degradation, the cytoplasmic IκBα pool is rapidly replenished by an accelerated production of the protein which is, at least in part, transcriptionally regulated (48) by the interaction of NF-κB with DNA-binding sites located in the promoter of the IκBα gene (14, 31).

The rapid reconstitution of the IκBα pool observed after degradation in stimulated cells led us to hypothesize that large amounts of newly synthesized IκBα could exceed the capacity of cytoplasmic NF-κB proteins to associate with the inhibitor,

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thus permitting free IκBα to accumulate in the nucleus. Although it is predominantly cytoplasmic, nuclear localization of IκBα has been previously reported (15, 51) when the protein is overexpressed from a transfected vector or microinjected into the cytoplasm. However, whether this phenomenon occurs in unmanipulated cells during normal physiological responses has not been documented.

In this work, we have investigated the presence of IκBα in the nuclei of HeLa S3 cells induced by either TNF or IL-1. We document that following IκBα degradation, nuclear localization of newly synthesized IκBα is a late event of cell activation mediated by both inducers. We have investigated the consequences of nuclear localization of IκBα for the regulation of the functional properties of NF-κB and the fate of nuclear NF-κB proteins.

MATERIALS AND METHODS

Cell culture. HeLa S3 cells were grown in suspension in minimal essential medium without calcium, containing 5% calf serum and supplemented with penicillin and streptomycin (culture medium).

Preparation of cytoplasmic and nuclear extracts. Cells were treated with 10 ng of TNF (obtained from the Medical Research Council AIDS Reagent Project) per ml cultivated in culture medium for 30 min at 37°C and then washed twice with 50 ml of prewarmed phosphate-buffered saline (PBS). After washes, aliquots of cells (10^7) were either resuspended in prewarmed culture medium in the absence of TNF (chase) or incubated with 10 ng of TNF per ml for an additional 90 min. When cycloheximide (CHX) (100 μg/ml) was incorporated into the cultures, it was added simultaneously with TNF at the onset of the induction and was maintained throughout the culture period. In experiments performed with IL-1 as the inducer, cells were maintained continuously for various times in the presence of 1 ng of recombinant IL-1β (a gift from A. Allison) per ml. In all cases, after treatment with either TNF or IL-1, cells were washed in PBS and collected by centrifugation. Sedimented cells were gently resuspended and disrupted for 1 min in 2 ml of lysis buffer containing Triton X-100 and protease inhibitors. After collecting 0.2 ml to prepare cytoplasmic extracts, 50 ml of lysis buffer was added to the remaining sample, which was then centrifuged to sediment nuclei. After careful removal of the supernatant, nuclear pellets were resuspended and maintained with permanent agitation for 1 h at 4°C in 200 μl of a hyperosmotic buffer containing protease inhibitors. Soluble fractions extracted from the nuclear suspension were recovered after centrifugation at 13,000 × *g* for 15 min. The composition of the lysis buffer was 50 mM NaCl, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 8.0]), 500 mM sucrose, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, and 0.2% Triton X-100. The hypertonic buffer was composed of 350 mM NaCl, 10 mM HEPES (pH 8.0), 25% glycerol, 0.1 mM EDTA, 0.5 mM spermidine, and 0.15 mM spermine. The protease inhibitors phenylmethylsulfonyl fluoride (1 mM), leupeptin (10 μM), pepstatin (1 μM), and L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butatone (50 μM) were included in the lysis and hypertonic buffers. To prepare nuclear extracts by using Ficoll cushions, cells were quickly disrupted with lysis buffer and layered over a buffer containing 10 mM HEPES (pH 7.8), 1 mM Mg_2Cl, 0.5 mM dithiothreitol, and 20% Ficoll. Samples were then spun for 10 min at 4,000 × *g* in a Sorvall HB-4 swing-out rotor. Supernatants were carefully discarded, and the nucleus pellet was resuspended in hypertonic buffer to extract nuclear proteins.

Lactate dehydrogenase activity (LDH) was determined to estimate contamination of nuclear extracts after cell fractionation. LDH was determined by the procedure of Frandsen and Schousboe (17). The assay contains pyruvate and NADH and measures the conversion of NADH to NAD^+ by its decrease in A_{340}. Protein samples were diluted to allow determination of initial rates. Values given are the change in optical density at 340 nm per minute per milligram of protein.

Immunoprecipitation of NF-κB-associated nuclear IκBα. Immunoglobulins from a preimmune rabbit serum or from anti-p65 or -p50 rabbit antisera were covalently cross-linked to protein A-Sepharose with dimethyl pimelimidate. Protein (200 to 400 μg) from nuclear cell extracts was incubated at 4°C with a 30-μl packed volume of antibody-coated beads. After centrifugation, protein A-Sepharose beads were washed 10 times with 35 volumes of PBS. Denaturation of immunoprecipitated proteins for Western blot (immunoblot) analysis was performed at room temperature in a solution containing 8 M urea and 3% sodium dodecyl sulfate (SDS).

Western blot analysis of cell fractions and immunoprecipitated proteins. Unless indicated otherwise, 40 μg of either cytoplasmic or nuclear protein from each sample was fractionated in an SDS–8% polyacrylamide gel, electrophoretically transferred to a nitrocellulose membrane, and probed with antibodies recognizing p50 or IκBα protein. To detect IκBα, affinity-purified antibodies from a rabbit polyclonal serum obtained by immunization with recombinant IκBα (36) or a murine monoclonal anti-IκBα antibody (a gift from K. Wood, Oxford, England) was used. p50 antibodies were affinity-purified immunoglobulins obtained from an immune polyclonal rabbit serum generated by immunization with recombinant p50. Horseradish peroxidase-labelled anti-rabbit or anti-mouse antibodies were used to detect specific antigen-antibody interactions by enhanced chemiluminescence (ECL kit; Amersham).

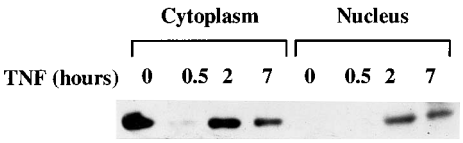

FIG. 1. Western blot analysis of TNF-induced HeLa S3 cells reveals the presence of nuclear IκBα. HeLa S3 cells, exposed to TNF for the indicated times, were fractionated into nuclei and cytoplasms. Proteins from cytoplasmic extracts (30 μg) or nuclear extracts (60 μg) were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and IκBα levels were determined with an affinity-purified antibody recognizing the IκBα protein.

Protein-DNA-binding assay. Nuclear extracts were analyzed in a gel electrophoresis DNA-binding assay with a [^{32}P]dCMP-labelled, double-stranded oligonucleotide containing the binding site for NF-κB (5′-CTGGGGACTTTC CACC-3′). Components of TNF-induced DNA-binding complexes were identified by incubation with either polyclonal antibodies directed against p50 or p65 recombinant proteins or 10 ng of recombinant IκBα (35), as previously described (4, 35). In competition experiments, a 40-fold molar excess of homologous unlabelled oligonucleotide was added.

Transfection of DNA plasmids. HeLa S3 cells were transfected at room temperature for 20 min with an HIV type 1 (HIV-1) long terminal repeat luciferase reporter (5) with 500 μg of DEAE-dextran per ml. Thirty minutes after the addition of TNF, transfected cells were washed extensively and incubation continued in a prewarmed culture medium, in either the absence or presence of 10 ng of TNF per ml. Cells were disrupted in a lysis buffer containing 1% Triton X-100, and luciferase activity in cell extracts was determined by luminometry. Data were corrected for the background reading obtained with a cell extract from nontransfected cells and are expressed as relative luciferase units per microgram of protein.

RESULTS

Newly synthesized IκBα accumulates in the nuclei of TNF- and IL-1-activated cells. Following exposure of HeLa S3 cells to TNF, the fate of IκBα was monitored by cell fractionation and Western blot analysis with affinity-purified anti-IκBα immunoglobulins. In agreement with previous reports (8, 13, 25, 37, 48), cytoplasmic IκBα was rapidly degraded, becoming virtually undetectable 30 min after induction with TNF. However, the cytoplasmic pool was replenished after 2 h and maintained for up to 7 h after the addition of TNF (Fig. 1). In the corresponding nuclear fractions, IκBα was undetectable prior to cell activation when the cytoplasmic levels of IκBα were high but was detected 2 and 7 h after exposure of cells to TNF.

To ascertain whether IκBα present in the nucleus represents an importation of newly synthesized material, cells were exposed to TNF for 30 min, washed, and cultured in either the absence (chase) or the presence of TNF. In both cases, incubation was prolonged for 90 min with or without the protein synthesis inhibitor CHX. As expected (Fig. 2), inhibition of protein synthesis by CHX prevented resynthesis of IκBα and nuclear accumulation of the protein. Densitometric analysis of chemiluminescent Western blot images revealed that a larger amount of newly synthesized IκBα accumulates in the cytoplasms and nuclei of cells exposed briefly to TNF and then maintained in its absence than in cells continuously exposed to the cytokine. Induction of HeLa S3 cells with IL-1 (Fig. 3a) proved that, following IκBα degradation, nuclear expression of IκBα can be obtained with kinetics similar to that observed in cells briefly treated with TNF.

The possibility that cytoplasmic material contaminated nuclear preparations was assessed by investigating the presence of p105 and other cytoplasmic markers in nuclear extracts of unstimulated and TNF-treated cells. Simultaneously, levels of IκBα in the two cell compartments were also measured. The

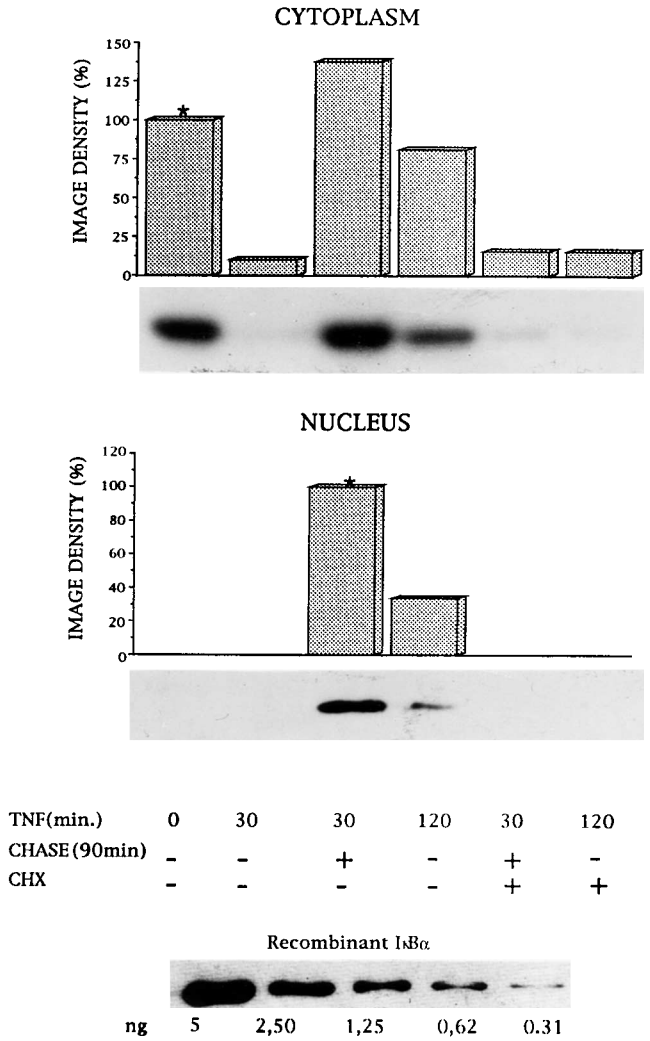

FIG. 2. Nuclear localization of newly synthesized IκBα occurs preferentially in cells briefly exposed to TNF and then maintained in its absence. After 30 min of exposure to TNF, cells were immediately washed and processed to obtain nuclear and cytoplasmic extracts or maintained in culture with or without (chase) TNF for an additional 90 min. When added, CHX was maintained throughout washes and the induction time. Forty micrograms of protein of each cytoplasmic or nuclear extract was analyzed by Western blotting with an affinity-purified anti-IκBα antibody. After the exposure of chemiluminescent blots, films were scanned and computer-generated images were analyzed with the National Institutes of Health IMAGE program to obtain densitometric values. For each series of samples (cytoplasmic or nuclear), the relative density of each image was calculated and expressed as a percentage of the value (arbitrarily set at 100) indicated by an asterisk. Images obtained from a chemiluminescent Western blot containing a twofold serial dilution of recombinant IκBα (5 to 0.31 ng) were used to asses the linearity of the densitometric values of this experiment. Values in pixels for the various amounts of recombinant IκB were as follows: 5 ng, 4,970; 2.5 ng, 2,300; 1.25 ng, 1,100; 0.62 ng, 550; and 0.31 ng, 220.

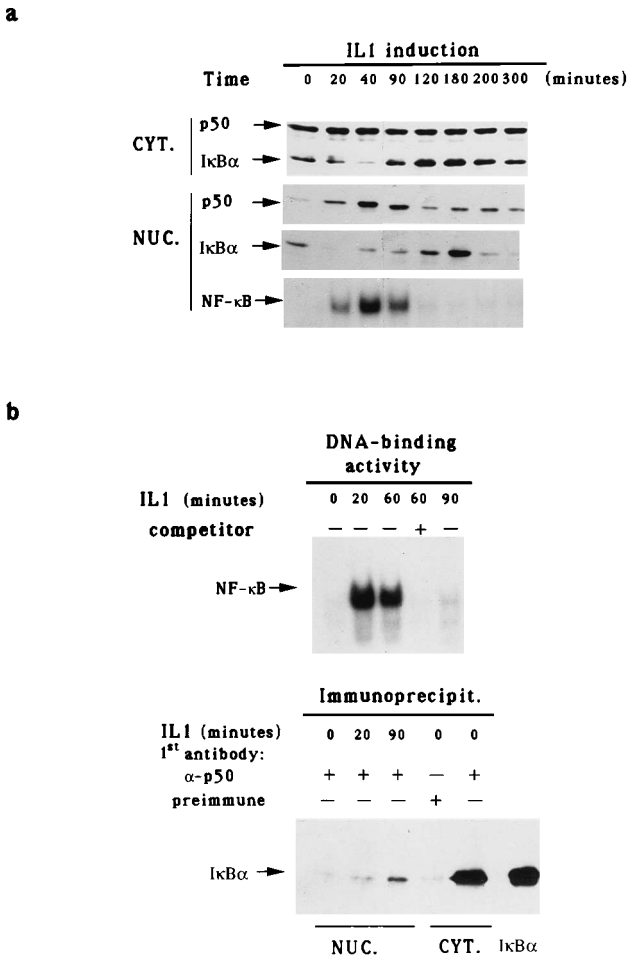

FIG. 3. Loss of IL-1-induced NF-κB DNA-binding activity correlates with the transient expression of nuclear IκBα. HeLa S3 cells were treated continuously for the indicated times with 1 ng of IL-1β per ml, and the expression of IκBα and p50 proteins in the nuclear (nuc.) and cytoplasmic (cyt.) compartments was analyzed simultaneously with the detection of nuclear NF-κB DNA-binding activity. (a) Western blot analysis of cytoplasmic and nuclear p50 and IκBα proteins (25 μg/sample). All the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels were blotted simultaneously, and membranes were probed first with anti-IκBα monoclonal antibody followed by an anti-p50 rabbit polyclonal serum. All the membranes were exposed simultaneously for chemiluminescence detection for 5 min. (b) Gel electrophoresis DNA-binding assay with 4 μg of nuclear proteins and a ^{32}P-labelled HIV enhancer oligonucleotide. Ten nanograms of unlabelled oligonucleotide (competitor) was used to prove the specificity of the DNA-binding activity detected. Immunoprecipitation of cell extracts revealed the existence of IκBα-p50 complexes concomitant with the disappearance of DNA-binding activity of NF-κB. Two hundred micrograms of each cytoplasmic or nuclear extract was incubated with anti-p50 immunoglobulins covalently cross-linked to agarose-protein A beads. Identically prepared immunoglobulins from the preimmune rabit serum were loaded and used as a control. Proteins were separated by SDS-PAGE and analyzed by Western blot analysis with a monoclonal anti-IκBα antibody. IκBα, 5 ng of recombinant protein.

results are shown in Fig. 4. Under experimental conditions that allowed detection of nuclear IκBα in cells briefly exposed to TNF and then maintained in its absence (Fig. 4a), the anti-p50 antibody revealed the presence of both p50 and p105 in the cytoplasm but not in nuclear extracts, where only p50 translocated from the cytoplasm was detected. Because of deliberate overexposure of the blots probed with anti-p50 antibody, some faint bands corresponding to proteins with an apparent molecular mass lower than 105 kDa were detected in nuclear but not in cytoplasmic extracts. In another independent experiment (Fig. 4b), analysis of nuclear extracts revealed, despite blot overexposure, the absence of a nonspecifically detected protein which remained virtually unaffected by either TNF induction or protein synthesis inhibition and which was exclusively detected in the cytoplasmic fraction when high concentrations of affinity-purified anti-IκBα were used. To ensure that equivalent amounts of nuclear extract were analyzed at each point, an identical blot was probed with antibodies to the SP1 transcription factor. The abundance of this factor in the nucleus did not change after TNF induction and was equivalent in nuclear

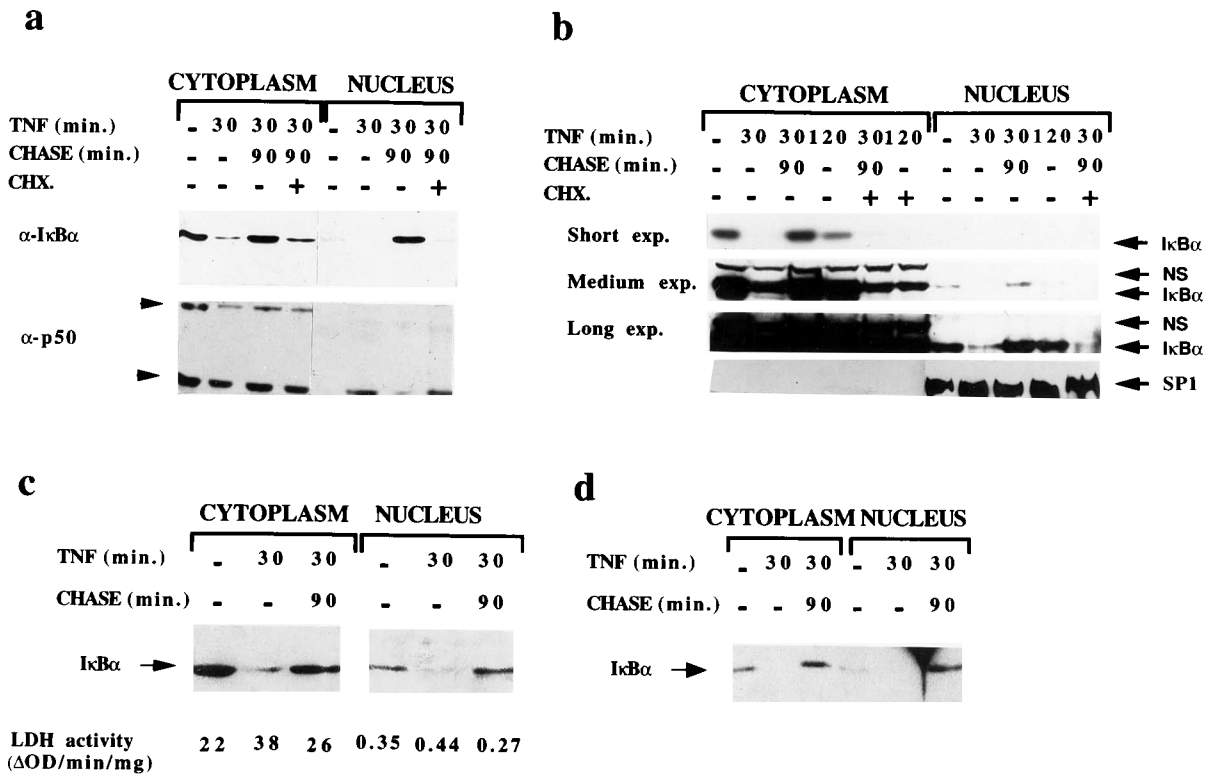

FIG. 4. Detection of nuclear IκBα, without cytoplasmic contamination, in cells briefly exposed to TNF and then maintained in its absence. (a) After 30 min of exposure to TNF, cells were immediately washed and processed to obtain nuclear and cytoplasmic extracts or maintained in culture either with or without (chase) TNF for an additional 90 min. When added, CHX was maintained throughout washes and the induction time. Forty micrograms of each sample of either cytoplasmic or nuclear extracts was analyzed by Western blotting with anti-IκBα (upper gels) or anti-p50 antibodies (lower gels). Lower and upper arrowheads indicate the positions of proteins with apparent molecular weights of 50,000 and 105,000, respectively, as estimated by the localization of bands corresponding to proteins detected by the anti-p50 antibody and the migration of prestained molecular weight markers. Cytoplasmic and nuclear samples were analyzed simultaneously, and for each antibody, blots were exposed for chemiluminescence for an identical length of time. Blots probed with anti-p50 were deliberately overexposed. (b) Nonspecifically detected protein species (NS) migrating more slowly than IκBα are exclusively cytoplasmic and are not detected by Western blot analysis in nuclear extracts even after long exposures. From top to bottom, the first three panels show the same blot probed with an affinity-purified anti-IκBα antibody and exposed for different lengths of time. The lowest panel shows immunodetection of the SP1 transcription factor, with a polyclonal antibody, in aliquots of cytoplasmic and nuclear samples processed and analyzed in parallel with the anti-IκBα Western blot described above. (c) Comparative analysis of IκBα detection (Western blot analysis) and LDH quantification to estimate the extent of cytoplasmic protein contaminating the nuclear preparations. LDH values are the change (△) in optical density at 340 nm per minute per milligram of protein. (d) Detection by Western blot analysis of IκBα in nuclear extracts obtained after detergent disruption of cells and centrifugation of lysates through a Ficoll pad.

extracts taken at all time points (Fig. 4b). A direct quantitation of cytoplasmic contaminants in the nuclear extracts was performed by using detection of the enzyme LDH as a marker. Data displayed in Fig. 4c show the lack of correlation between the residual LDH contamination in nuclear preparations (1 to 2%) and the amounts of IκBα detected in the respective nuclear extracts. Detection of IκBα in nuclear preparations obtained after centrifugation through a Ficoll cushion (Fig. 4d), which removes soluble contaminants, provided additional evidence that IκBα detected in nuclear extracts results from the presence of the inhibitor in the nuclei and does not merely reflect contamination by cytoplasmic IκBα. It should be noted that in some experiments nuclear IκBα was clearly detected prior to stimulation (Fig. 3a, 4c, and 4d). This may have been a consequence of low-level activation of the cells due to serum components of the medium, but this material is also degraded and the newly synthesized material accumulates in the nucleus as in other experiments in which IκBα prior to cell induction was not detected or barely detectable (Fig. 1 and 4a, respectively).

Nuclear expression of IκBα occurs concomitantly with the loss of NF-κB DNA-binding activity in activated cells. To investigate the consequences of TNF- and IL-1-induced nuclear translocation of IκBα, the DNA-binding activity of NF-κB was determined by gel electrophoresis DNA-binding assay (Fig. 3b and 5a). NF-κB DNA-binding activity was not detected in unstimulated cells but was clearly detectable after the addition of TNF (30 min) or IL-1 (20 min) (Fig. 3b and 5a). In cells continuously exposed to TNF, the level of NF-κB DNA-binding activity remained high, despite a slight reduction, after continued exposure for 120 min (Fig. 5a, lane 5). However, if the cytokine was removed after 30 min and incubation continued in the absence (chase) of TNF for a further 90 min (Fig. 5a, lane 3), NF-κB DNA-binding activity was dramatically reduced. Loss of nuclear DNA-binding activity in cells briefly exposed to TNF and then maintained in its absence was concomitant with the presence of IκBα in the nuclear compartment (Fig. 2 and 4a). The addition of CHX (Fig. 5a, lane 4) during this chase period prevented the reduction in DNA-binding activity, in keeping with the inhibition of IκBα synthesis (Fig. 2 and 4a). In cells continuously exposed to IL-1, nuclear expression of NF-κB DNA-binding activity was still detectable 60 min after induction but was dramatically reduced at later times (Fig. 3a and b). As with cells treated for 30 min with TNF and then cultured in its absence, progressive inhibition of NF-κB-binding activity was observed in IL-1-induced

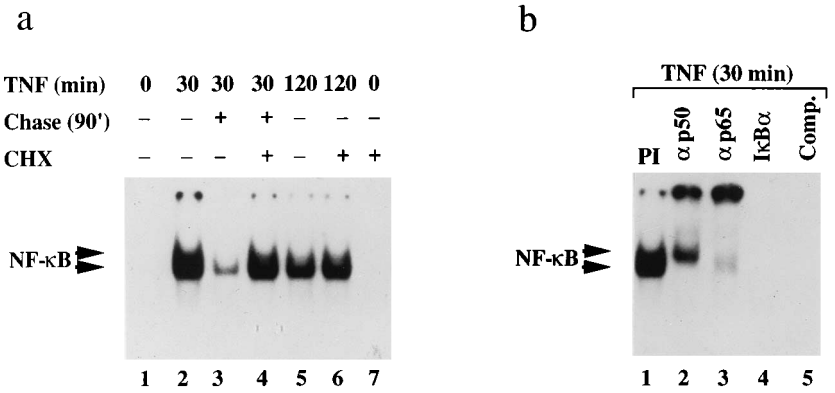

FIG. 5. Nuclear IκBα inhibits the DNA-binding activity of NF-κB. (a) Eight micrograms of each nuclear extract was analyzed in a gel electrophoresis DNA-binding assay with a ^{32}P-labelled HIV enhancer oligonucleotide as described in the text. (b) αp65 and αp50, specific polyclonal rabbit antisera; PI, rabbit preimmune serum. Rabbit sera (1 μl) or 10 ng of recombinant IκBα was incubated for 15 min with the indicated samples in a final volume of 20 μl prior to addition of the radiolabelled probe. Comp., unlabelled HIV enhancer oligonucleotide (20-fold molar excess) added to sample in lane 2 of panel a prior to the addition of ^{32}P-labelled probe.

cells (Fig. 3a and b), at the times when IκBα progressively accumulates in the nuclear compartment (Fig. 3a).

Specific competition by unlabelled DNA (Fig. 3b and 5b, lane 5), recognition by p50 and p65 antibodies (Fig. 5b, lanes 2 and 3), and inhibition by recombinant IκBα protein (Fig. 5b, lane 4) indicated that the TNF-induced DNA-binding activity (Fig. 5b, lane 1) was composed, at least in part, of bona fide (p50-p65 subunits) NF-κB. However, the failure of anti-p50 antibody (and antibodies to p50B, c-rel, and relB [data not shown]) to achieve a complete upshifting of the more slowly migrating complex (Fig. 5b) is compatible with the existence, along with the p50-p65 complex, of either p65 homodimers or heterodimers of p65 and an unrecognized protein.

IκBα–NF-κB complexes are detected in the nuclei of TNF- and IL-1-activated cells. To directly demonstrate that the newly synthesized, nuclear IκBα is bound to NF-κB, antibodies specific for the p50 or the p65 subunits of NF-κB were used to coimmunoprecipitate IκBα, which was then detected by Western blotting (Fig. 6a and b, respectively). This procedure revealed a physical association between components of NF-κB and IκBα in nuclear extracts from cells briefly exposed to TNF and then maintained in its absence (Fig. 6a, lane 3, and 6b, lane 2) and, to a lesser extent, in cells exposed to TNF continuously for 120 min (Fig. 6a and b, lanes 4). NF-κB-associated IκBα was not detected in nuclei of unstimulated cells (Fig. 6a, lane 1), nor was it detectable after 30 min of TNF induction (Fig. 6a, lane 2, and 6b, lane 1) since IκBα was degraded. When CHX was used to block de novo protein synthesis, detection of IκBα in the coimmunoprecipitated material was greatly impaired (Fig. 6b, lanes 3 and 5). To assess the specificity of the immunoprecipitations performed in nuclear extracts shown in Fig. 6a (lanes 1 to 4) and 6b (lanes 1 to 5), immunoglobulins from preimmune sera were incubated with cytoplasmic extracts from unstimulated cells, in which most of IκBα is supposed to be associated with NF-κB. Large amounts of IκBα were coprecipitated with specific antibodies (Fig. 6a and b, lanes 5 and 7, respectively) but not with preimmune immunoglobulins (Fig. 6a and b, lanes 6). As described above for cells briefly exposed to TNF and then maintained in its absence, loss of NF-κB DNA-binding activity in cells continuously exposed to IL-1 for 90 to 120 min was concomitant with the presence of nuclear IκBα associated with NF-κB (Fig. 3a and b). In both IL-1-treated cells and cells treated briefly with TNF, maximal amounts of IκBα were immunoprecipitated by anti-p50 antibodies when reduced levels of p50 were detected in cell nuclei (Fig. 3a and b; Fig. 6a and c, lanes 3). This apparent paradox could be explained by the fact that most of the remaining p50 protein was associated with IκBα, which was progressively accumulating in the nuclei as the levels of p50 decreased (Fig. 3a). Although the levels of nuclear p50 are low at that time, the immunoprecipitation step serves to concentrate the p50 protein, allowing detection of associated IκBα by Western blotting.

Coincident with the expression of nuclear IκBα in cells briefly exposed to TNF, it was observed by Western blot analysis that the total content of nuclear p50 protein was greatly reduced (Fig. 6c; see also Fig. 4a, lower right gel). Indeed, p50 accumulated rapidly (30 min) in the nucleus after exposure to TNF (Fig. 6c, lane 2), but its level was markedly reduced by further incubation in the absence (chase) of TNF (lane 3). The reduction of nuclear p50 content was prevented by CHX (Fig. 6c, lane 5) coincident with the inhibition of synthesis and reexpression of IκBα in both cytoplasmic and nuclear compartments (Fig. 2 and 4a). Additional evidence of the loss of p50 proteins following colocalization to the nucleus of NF-κB and IκBα expression was provided by the kinetic analysis of NF-κB activation induced by IL-1 (Fig. 3a). Indeed, following maximal expression of p50 in the nucleus, which tightly paralleled the level of NF-κB DNA-binding activity, the total amount of nuclear p50 declined drastically as increased amounts of IκBα accumulated in the nuclear compartment. Furthermore, accumulation of IκBα was also transient, and continued incubation resulted in a progressive reduction of the nuclear content of the inhibitor. Detection of nuclear p50-IκBα complexes at this point supports the notion that reduction of p50 nuclear content occurs after NF-κB dissociates from DNA following interaction with the inhibitor.

Nuclear expression of IκBα correlates with inhibition of NF-κB-dependent transcription. To investigate the functional relevance of reduced NF-κB-binding activity which occurs concomitantly with the nuclear expression of IκBα, transient-transfection experiments were performed with an NF-κB-responsive (HIV-1 long terminal repeat) luciferase reporter plasmid (Fig. 7). Transfected HeLa S3 cells were either incubated continuously in the presence of TNF or exposed to TNF for 30 min and then further incubated in its absence. After 2 h, luciferase activity was equivalent in both sets of cells, but after 6 h, cells incubated continuously in the presence of TNF had a fourfold-higher level of luciferase activity than cells exposed to TNF for 30 min and then incubated for 5.5 h in the absence of

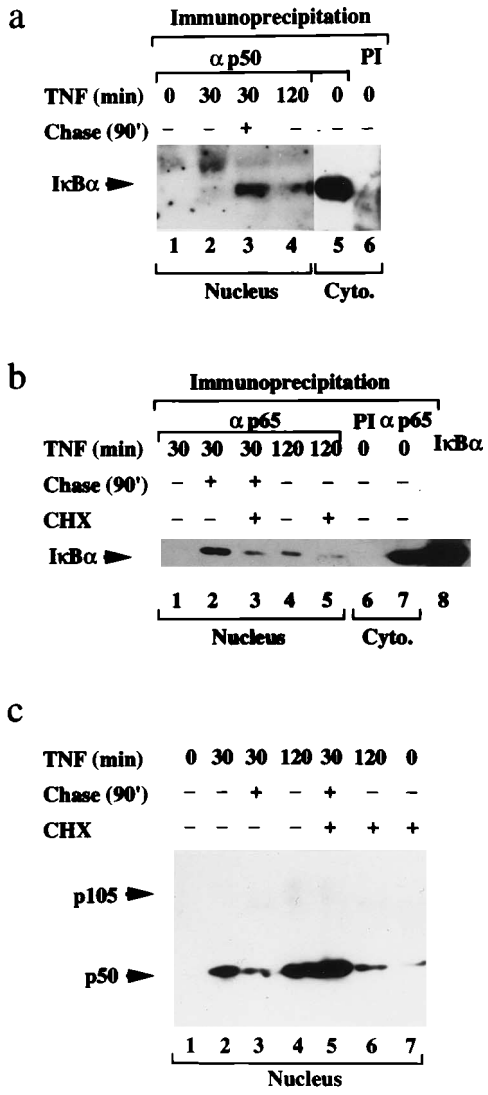

FIG. 6. IκBα expressed in the nuclei of TNF-activated cells is bound to NF-κB. (a and b) Results from two different experiments are shown. Nuclear and cytoplasmic extracts (400 μg of protein of each sample) were incubated with either anti-p50 (αp50 [a]) or anti-p65 (αp65 [b]) rabbit immunoglobulins covalently cross-linked to protein A-Sepharose. After immunoprecipitation, the presence of IκBα was analyzed by Western blot analysis with a murine monoclonal anti-IκBα antibody. In panels a and b, identical amounts (400 μg) of cytoplasmic extract from unstimulated cells were used to demonstrate the specificity of the immunoprecipitations with protein A-Sepharose-cross-linked immunoglobulins from preimmune sera (PI), under experimental conditions in which large amounts of IκBα associated with NF-κB can be coimmunoprecipitated. In panel a, images shown in lanes 5 and 6 were obtained from a less exposed film to reduce the intensity of the band detected in lane 5, a consequence of the large amount of IκBα coprecipitated with the anti-p50 antibody from the cytoplasmic cell extract. In panel b, 10 ng of recombinant IκBα (lane 8) was loaded. (c) The amount of p50 expressed in nuclear extracts corresponding to the experiment shown in panel a was investigated by Western blot analysis with affinity-purified antibody.

TNF. Similar results were observed when a luciferase expression vector carrying a conalbumin promoter and driven by three synthetic copies of the NF-κB consensus motif from the κ light chain gene promoter (a gift from A. Israël) was used (data not shown). A reporter plasmid in which the NF-κB-binding sites had been deleted was not responsive to TNF (data not shown).

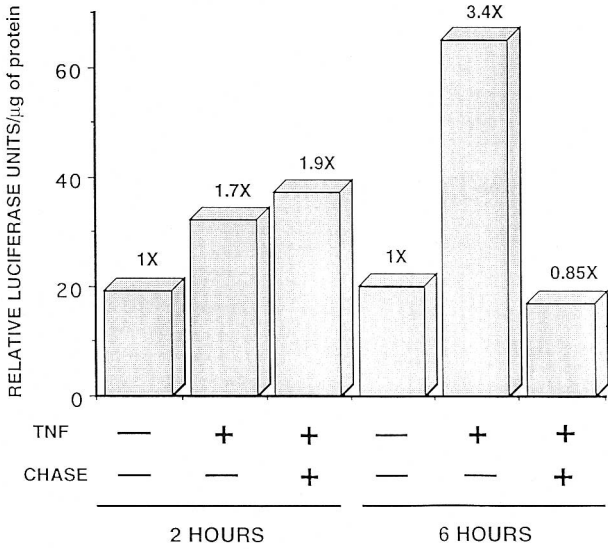

FIG. 7. Negative regulation of NF-κB-dependent transcription in cells briefly exposed to TNF and then maintained in its absence. HeLa S3 cells were transfected with a luciferase reporter placed under the control of a wild-type HIV-1 long terminal repeat. After 24 h in culture, cells were treated for 30 min with TNF (10 ng/ml). Untreated and TNF-treated cells were extensively washed in prewarmed PBS and reseeded in fresh culture medium in either the absence (chase) or presence of TNF. Total cell extracts were analyzed for luciferase activity 2 and 6 h after the addition of TNF. The numbers above the bars indicate fold amplification of luciferase activity relative to the enzymatic activity in untreated cells. The experiment was repeated twice with identical results.

DISCUSSION

Using Western blot analysis we showed that newly synthesized IκBα localizes to the nucleus of either TNF- or IL-1-activated cells, where it inhibits DNA binding and the transcriptional activity of NF-κB. Attempts to detect in situ localization of IκBα by immunofluorescence, in either unstimulated or TNF-activated cells, were unsuccessful as the staining pattern was not consistently above the background level. In contrast, and in agreement with previous reports (15, 51), IκBα was clearly detected when overexpressed from a transfected expression vector (data not shown). The relative paucity of IκBα therefore makes in situ detection of the protein in nontransfected cells difficult.

Although nuclear translocation of IκBα is detected in cells continuously exposed to TNF, we observed maximal nuclear and cytoplasmic accumulation of IκBα in cells briefly exposed to TNF and then maintained in its absence (see Fig. 2). The reasons for such a phenomenon are not completely understood, but it could reflect a decreased proteolysis of newly synthesized IκBα in cells transiently treated with TNF compared with that in cells continuously exposed to this inducer. These observations and the fact that transient exposure to TNF may mimic physiological mechanisms of cell activation prompted us to adopt this system. A number of arguments support the physiological relevance of cell activation induced upon a brief exposure to TNF. Indeed, recombinant TNF induces septic shock-like syndromes when administered to animals despite a half-life estimated to be only minutes (10). Additionally, transient release of TNF followed by rapid decline to basal levels has been observed upon injection of endotoxin into humans and animals (10, 38). Furthermore, in in vivo situations, the participation of TNF in inflammatory reactions could be restricted by the suppression of TNF production by IL-10 (19, 34) and/or the interaction with soluble TNF

receptors (2, 46). In all these situations a net negative regulation of NF-κB functions by nuclear IκBα may represent an important regulatory mechanism. The situation described above is not a peculiarity of TNF induction, since the rapid decline of NF-κB DNA-binding activity observed upon IL-1 stimulation was also concomitant with the localization of IκBα, associated with NF-κB subunits, in the nuclear compartment.

IκBα detected in nuclear extracts is a direct result of nuclear localization of the protein and is not merely a reflection of contamination by cytoplasmic IκBα. This assertion is supported by a number of observations. The protein was detected even when nuclear extracts were virtually free of cytoplasmic material as determined by measurement of cytoplasmic p105, LDH, or another nonspecific protein. Nuclear IκBα was also detected in detergent-treated nuclei, lacking both inner and outer nuclear membranes (1, 3), which had been collected by centrifugation through a Ficoll cushion. Finally, in kinetic analysis following IL-1 induction it was evident that the amount of neither IκBα nor p50 present in the nuclei paralleled the levels of the corresponding cytoplasmic proteins at the time when DNA-binding activity of NF-κB decreased progressively (Fig. 3a).

Analysis of nuclear extracts from cells briefly exposed to TNF and then maintained in its absence revealed a dramatic reduction of NF-κB binding similar to that previously published by others (13). The striking correlation observed between the reduction of NF-κB binding and the expression of nuclear IκBα was strengthened by the fact that CHX prevented reexpression of IκBα and maintained NF-κB-binding activity at a level comparable to that detected 30 min after TNF induction, when IκBα was undetectable in both cytoplasmic and nuclear cell extracts. We speculate that after TNF treatment, the large amount of IκBα generated may exceed the capacity of NF-κB subunits to anchor IκBα in the cytoplasm. Additionally, posttranslational modifications undergone upon cell activation, such as phosphorylation of either IκBα or NF-κB subunits (24, 37), could reduce their capacity to associate in the cytoplasm, allowing newly synthesized IκBα to accumulate in the nucleus. Accumulation of newly synthesized IκBα in the nuclear compartment in IL-1-induced cells was observed even though the cytokine was not removed from the cell cultures. This phenomenon was not exclusive of HeLa S3 cells and was also detected in U937 monocytoid cells concomitantly with the decline of NF-κB DNA-binding activity (43a). The precise mechanism underlying this phenomenon has not been elucidated and deserves further investigation.

The mechanism by which IκBα is exported across the nuclear membrane remains to be determined. It has been proposed that the small size (37 kDa) of the protein might allow IκBα expressed from transfected vectors to penetrate into the nucleus independently of an NLS sequence (51). Alternatively, by interacting with proteins bearing an NLS (12), IκBα could be transported from the cytoplasm into the nucleus. Finally, it is also possible that IκBα contains an as-yet-unrecognized NLS that accounts for its ability to rapidly accumulate in the nucleus.

The amount of IκBα coimmunoprecipitated with p50 or p65 proteins was inversely correlated with the intensity of NF-κB-binding activity detected in the nuclei of cells briefly exposed to TNF and then maintained in its absence. The lack of p50 homodimers and the fact that anti-p65 antibody removed faster-migrating DNA-binding complexes containing p50 indicated that most of IκBα associated with p50 is probably part of heterotrimeric complexes including p65, although we cannot exclude the possibility that part of IκBα associated with p65 is integrated into protein complexes other than bona fide (p50-p65) NF-κB. These results indicate that newly synthesized IκBα in either IL-1- or TNF-activated cells is capable of binding nuclear NF-κB complexes. IκBα was also coimmunoprecipitated, albeit to a lesser extent, with either anti-p50 or anti-p65 antibodies from the nuclei of cells continuously exposed to TNF for 2 h. In this case, the reduced accumulation of nuclear IκBα compared with that in cells briefly exposed to TNF (Fig. 2) and a continuous translocation of NF-κB to the nucleus could account for the modest reduction in NF-κB DNA-binding activity that was evident in cells continuously exposed to TNF.

Taking advantage of the approximately 3-h half-life of luciferase (39), we have shown that, in contrast to cells continuously treated with TNF, cells briefly exposed to TNF and then maintained in its absence show an accumulation of IκBα in the nucleus and a concomitant decrease in NF-κB-induced luciferase activity. It seems reasonable to postulate that the interaction of NF-κB with IκBα, and the corresponding inhibition of DNA binding, may account for the extinction of luciferase transcription.

When the nuclear content of p50 was evaluated in cells briefly exposed to TNF and then maintained in its absence for an additional 90 min, we were surprised to find a substantial decrease in the level of this subunit. The reproducibility of the phenomenon and the fact that maximal nuclear IκBα accumulation was detected in the same protein extracts excluded loss of nuclear material in these samples. Inhibition of protein synthesis prevented the reduction of nuclear p50, suggesting that the absence of newly synthesized IκBα in the nuclei accounted for the high levels of nuclear p50 observed in CHX-treated cells. The precise mechanisms underlying this phenomenon are not understood. However, results obtained with IL-1-treated cells confirmed and extended these observations, strengthening the likely physiologic relevance of the phenomenon. The possibility that p50 becomes susceptible to proteolysis in the nucleus when it is associated with IκBα and p65 is unlikely, although it cannot be formally ruled out. A more attractive hypothesis would be that when bound to IκBα, p50-p65 complexes can be transported in a retrograde fashion to the cytoplasm. This model is supported by the following observations. First, we show that upon cell activation, newly synthesized IκBα localizes in the nucleus. Second, it is well established that IκB proteins interact with the NLS of NF-κB such that the NLS are functionally occluded; p65 containing a functional NLS is located cytoplasmically when coexpressed with IκBα (51). Third, it has been shown that proteins are exported from the nucleus by a default mechanism, unless they contain a functional NLS (30, 45). Thus, it is likely that the masking of the NF-κB NLS by IκBα (or mutual masking of NF-κB and a hypothetical IκBα NLS) leads to transport of the protein complex back to the cytoplasm, where it can respond to new cellular activation signals.

In light of the present results, it appears that cellular activation results in both an early translocation of NF-κB into the nucleus and a later event negatively regulating NF-κB transcription activity, via synthesis and nuclear accumulation of IκBα. Nuclear IκBα inhibits the DNA-binding activity of NF-κB, thereby contributing to the extinction of ongoing NF-κB-dependent transcription. Furthermore, our results are also consistent with the notion that NF-κB and IκBα could be transported in a retrograde fashion to the cytoplasm.

ACKNOWLEDGMENTS

This work was supported by the MRC AIDS Directed Programme (United Kingdom), Agence Nationale pour la Recherche sur le SIDA (ANRS) (France), and European Communities Concerted Action

(Project ROCIO). F.A.-S. was supported by an MRC/INSERM exchange fellowship, J.T. was supported by the Leukaemia Research Fund, and F.B. was supported by ANRS.

We thank John Nicholson for providing HeLa cells and Jean Louis Virelizier, Susan Michelson, and J. M. Cavaillon for helpful discussions and critical reading of the manuscript. We are also indebted to R. Griffiths and A. Grieve for helping us in LDH measurements.

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