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66. Note that the situation for catalytic antibodies is probably the converse of that for enzymes. For enzymes, transition-state analog binding is less than expected on the basis of their very great catalytic accelerations. For the antibodies, catalytic accelerations are less than expected on the basis of their very strong transition-state analog binding. The reason is the same in both cases: analogs are not really transition states. Nevertheless, Dr. Johnson's simile is apropos: "... like a dog's walking on his hind legs. It is not done well; but you are surprised to find it done at all."
67. For purposes of comparison note that the phosphorus-containing moiety here is a phosphoric acid diester, in which a central phosphorus atom is tetrahedrally bonded to four oxygen atoms, whereas the phosphonic acid esters and amides featured in the preceding and following discussion are organophosphorus compounds in which a phosphorus atom is tetrahedrally bonded to carbon and three heteroatoms.

Research Articles

IκB: A Specific Inhibitor of the NF-κB Transcription Factor

PATRICK A. BAEUERLE AND DAVID BALTIMORE

In cells that do not express immunoglobulin kappa light chain genes, the kappa enhancer binding protein NF-κB is found in cytosolic fractions and exhibits DNA binding activity only in the presence of a dissociating agent such as sodium deoxycholate. The dependence on deoxycholate is shown to result from association of NF-κB with a 60- to 70-kilodalton inhibitory protein (IκB). The fractionated inhibitor can inactivate NF-κB from various sources—including the nuclei of phorbol ester–treated cells—in a specific, saturable, and reversible manner. The cytoplasmic localization of the complex of NF-κB and IκB was supported by enucleation experiments. An active phorbol ester must therefore, presumably by activation of protein kinase C, cause dissociation of a cytoplasmic complex of NF-κB and IκB by modifying IκB. This releases active NF-κB which can translocate into the nucleus to activate target enhancers. The data show the existence of a phorbol ester–responsive regulatory protein that acts by controlling the DNA binding activity and subcellular localization of a transcription factor.

IN EUKARYOTIC CELLS, THE RATE OF TRANSCRIPTION OF MANY genes is altered in response to extracellular stimuli. Changes in expression of genes transcribed by RNA polymerase II in response to such agents as steroid hormones, growth factors, interferon, tumor promoters, heavy metal ions and heat shock are mediated through distinct cis-acting DNA-sequence elements (*1*). Most important are those called enhancers (*2*), which display great positional flexibility with respect to the gene they control (*3*), and promoters, which are confined to the 5′ noncoding region of the gene (*4*). Both cis-acting elements contain multiple binding sites for sequence specific DNA-binding proteins (*1, 5*). The demonstration of protein-DNA interaction in vivo (*6*), competition experiments in vitro (*7*) and in vivo (*8*), and the definition of protein binding sites by mutational alteration of regulatory DNA sequences (*9, 10*) suggested that occupation of cis-acting elements by trans-acting factors is crucial for the transcriptional activity of constitutive and inducible genes. There is increasing evidence that inducible transcription of genes is mediated through induction of the activity of

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trans-acting protein factors (*11–14*). Possible mechanisms include the transcriptional activation of genes encoding trans-acting factors, the activation of DNA binding activity from an inactive precursor, inducible nuclear translocation, and modification of an already DNA-associated factor to acquire transcription activating competence.

The NF-κB transcription factor provides a model system to study the activation of a phorbol ester–inducible transcription factor. Binding of NF-κB to the κ light chain enhancer, the human immunodeficiency virus (HIV) enhancer, and an upstream sequence of the interleukin-2 receptor α-chain gene has been shown to confer transcriptional activity and phorbol ester inducibility to genes controlled by these cis-acting elements (*9, 15*). Mutations in the binding sites for NF-κB abolish these effects. Oligonucleotides representing NF-κB binding sites inserted in either upstream or downstream enhancer (*16*) or promoter positions (*17*) are sufficient to confer phorbol ester–inducible and cell stage–specific transcriptional activity to test genes. In most cell lines, except for those derived from mature B cells, DNA binding activity of NF-κB is not evident in nuclear extracts unless cells have been treated with the phorbol ester TPA (12-*O*-tetradecanoylphorbol 13-acetate) (*11, 18*). This activation of NF-κB is independent of new protein synthesis, suggesting that NF-κB is present in most cell types as an inactive precursor before TPA stimulation.

We showed earlier that the NF-κB transcription factor can be detected in two forms (*19*). One form, in nuclear extracts from TPA-stimulated cells, does not require dissociating agents for DNA binding activity. The other form fractionates into the cytosol of unstimulated cells and is only active in the presence of dissociating agents such as sodium deoxycholate (DOC) or after denaturation and electrophoretic fractionation. The conversion of inactive into active NF-κB by TPA in vivo or by dissociating agents in vitro and the different subcellular fractionation of the two forms of NF-κB suggested that the activation of NF-κB takes place in at least two steps, namely, derepression of DNA binding activity and nuclear translocation.

To investigate whether the DOC-dependence of cytosolic NF-κB results from its association with an inhibitor, we probed for an activity in cytosolic fractions that would specifically prevent DNA binding to NF-κB in electrophoretic mobility shift assays (EMSA). Here we demonstrate a protein inhibitor (called IκB) in cytosolic fractions of unstimulated pre-B cells that can convert NF-κB into an inactive DOC-dependent form by a reversible, saturable, and specific reaction. The inhibitory activity becomes evident only after selective removal of the endogenous cytosolic NF-κB under dissociating conditions, suggesting that NF-κB and IκB were present in a stoichiometric complex. Enucleation experiments showed that the complex of NF-κB and IκB is truly cytoplasmic. Our data are consistent with a molecular mechanism of inducible gene expression by which a cytoplasmic transcription factor–inhibitor complex is dissociated by the action of TPA presumably through activation of protein kinase C. The dissociation event results in activation and apparent nuclear translocation of the transcription factor. It would appear that IκB is the target for the TPA-induced dissociation reaction.

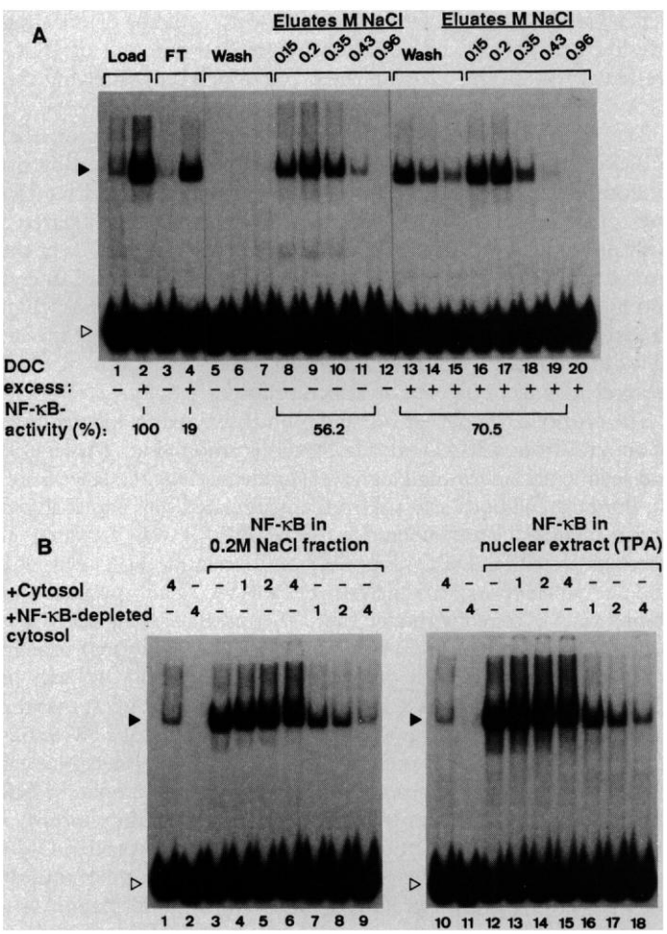

Fig. 1. DNA-cellulose chromatography of DOC-treated cytosol. Cytosol was prepared from unstimulated 70Z/3 pre-B cells (*19*) and protein concentrations were determined (*40*). In the fluorograms of native gels shown, the filled arrowheads indicate the position of the NF-κB–κ enhancer fragment complex (*19*), and the open arrowheads indicate the position of unbound DNA probe. (**A**) Release of DOC-independent NF-κB activity. Equal proportions of load, flow-through (FT), washings, and eluates (*41*) were analyzed by EMSA (*18, 19*) with (+) or without (–) excess DOC (*41*). The ^{32}P radioactivity in the NF-κB–DNA complexes was counted by liquid scintillation and the percentage of NF-κB activity recovered in the various fractions was calculated. (**B**) Release of an inhibitory activity. NF-κB contained in the 0.2*M* NaCl fraction (31 ng of protein) or NF-κB in a nuclear extract from TPA-treated 70Z/3 cells (1.1 μg of protein) was incubated under nondissociating conditions (*41*) with the indicated amounts (in microliters) of either cytosol which was DOC-treated but not passed over DNA-cellulose (lanes 4 to 6 and 13 to 15) or the flow-through fraction (referred to as NF-κB–depleted cytosol; lanes 7 to 9 and 16 to 18).

Separation of an inhibitor from NF-κB. We examined cytosolic fractions from unstimulated 70Z/3 pre-B cells for an activity that would impair the DNA binding activity of added NF-κB in an EMSA (*18, 19*). Increasing amounts of cytosol from unstimulated cells did not significantly influence the formation of a protein-DNA complex between NF-κB and a κ enhancer fragment (Fig. 1B, lanes 13 to 15). This indicated the absence of free inhibitor, presumably because all of it is complexed with endogenous NF-κB. In an attempt to liberate the inhibitor, we used DNA-cellulose to selectively remove the endogenous NF-κB from DOC-treated cytosol. Almost all NF-κB was present in a DOC-dependent form prior to DOC activation and chromatography (Fig. 1A, lanes 1 and 2). In the presence of excess DOC, about 80 percent of the NF-κB activity was retained by DNA-cellulose (Fig. 1A, compare lanes 2 and 4) most of which eluted from the DNA-cellulose between 0.15 and 0.35*M* NaCl (Fig. 1A, lanes 8 to 10 and 16 to 18). The NF-κB activity eluting at high salt concentration was detectable in mobility shift assays in the absence of excess DOC (Fig. 1A, lanes 8 to 11), indicating that NF-κB had been separated from an activity that caused its DOC-dependent DNA binding activity. In contrast, the small percentage of NF-κB activity contained in the washings was still dependent on DOC (Fig. 1A, compare lanes 5 to 7 and 13 to 15). These results show that affinity chromatography is sufficient to convert DOC-dependent NF-κB precursor into DOC-independent

active NF-κB, similar to that found in nuclear extracts from TPA-stimulated cells.

The flow-through fraction from the DNA-cellulose was assayed for an activity that, after neutralization of DOC by non-ionic detergent, would inactivate added NF-κB from the 0.2*M* NaCl fraction from nuclear extracts of TPA-stimulated cells. Increasing amounts of cytosol from which the endogenous NF-κB was removed inhibited the formation of an NF-κB–DNA complex as monitored by EMSA (Fig. 1B, lanes 7 to 9 and 16 to 18). The DOC-treated cytosol that was not passed over DNA-cellulose had no effect (Fig. 1B, lanes 4 to 6 and 13 to 15) even if cells had been treated with TPA (*20*). Because, after DNA-cellulose chromatography of DOC-treated cytosol, we found both DOC-independent NF-κB activity and an inhibitory activity, we believe that we separated NF-κB from an inhibitor. We refer to this inhibitor as IκB.

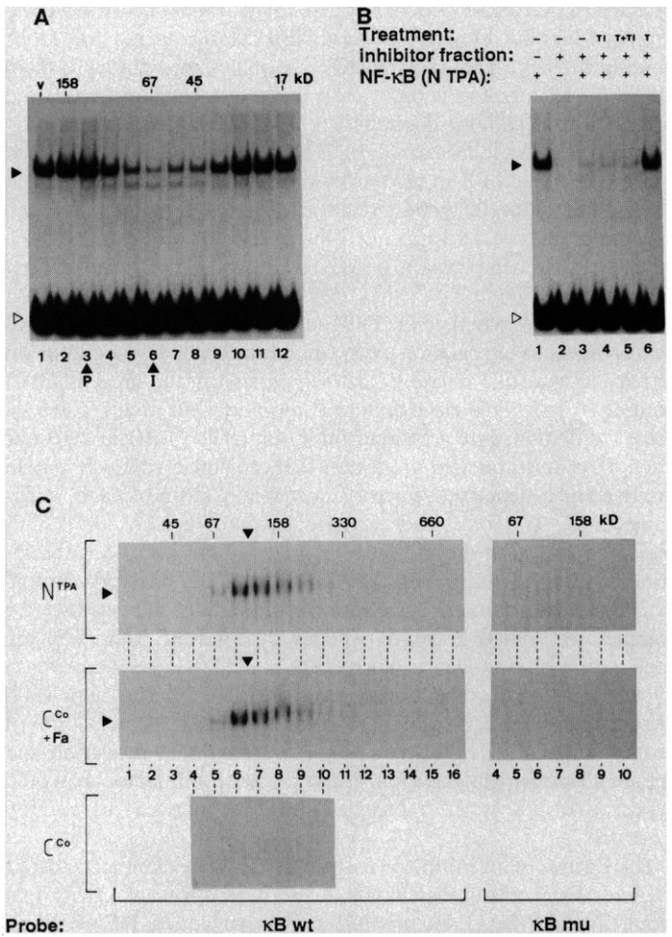

Fig. 2. Characterization of IκB and its complex with NF-κB. In the fluorograms shown, the filled arrowheads indicate the position of the NF-κB–DNA complex and the open arrowheads the position of free DNA probe. (**A**) For size determination of IκB, the flow-through from the DNA-cellulose column (*41*) was passed over a G-200 Sephadex column (*42*). Portions of fractions were incubated with NF-κB contained in nuclear extracts from TPA-stimulated 70Z/3 cells (N TPA) (*43*), and analyzed by EMSA [v, void volume; P, fraction where remaining NF-κB precursor (Fig. 1A, lane 4) peaked after gel filtration as assayed with excess DOC in the absence of added NF-κB; I, fraction where the inhibiting activity peaked]. (**B**) The effect of trypsin treatment (*45*) on the inhibiting activity of IκB. NF-κB in a nuclear extract (lane 1) was incubated with a fraction containing inhibitor (lane 2) without any addition (−; lane 3) or with bovine pancreas trypsin inhibitor (TI; lane 4), trypsin that had been incubated with BPTI (T + TI; lane 5), or with trypsin alone (T; lane 6). Samples were then used in the inhibitor assay (*43*). (**C**) Glycerol gradient sedimentation of NF-κB and its complex with IκB. Nuclear extract from TPA-stimulated 70Z/3 cells (N TPA) and cytosol from unstimulated cells (C Co) were subjected to sedimentation through a glycerol gradient (*46*). Cosedimented size markers were ovalbumin (45 kD), BSA (67 kD), immunoglobulin G (158 kD) and thyroglobulin (330 and 660 kD). NF-κB activity was detected in the fractions by EMSA with a wild-type κ enhancer fragment (κB wt, left panels). The specificity was tested with a mutant fragment (*9*) (κB mu, right panels). The inactive cytosolic NF-κB precursor (lower panel) was activated by formamide treatment (*19*) (Fa; middle panel).

IκB fractionates as a 60- to 70-kD protein. The flow-through fraction from the DNA-cellulose column was subjected to gel filtration through G-200 Sephadex, and the fractions were assayed for an activity that would interfere with the DNA binding activity of added NF-κB contained in a nuclear extract from TPA-stimulated 70Z/3 cells (Fig. 2A). The 67-kD fraction had the highest activity; it virtually completely prevented interaction of NF-κB and DNA (Fig. 2A, lane 6). In fractions from a G-75 Sephadex column, no additional inhibitor of low molecular size was detectable (*20*), an indication that NF-κB was inactivated by a macromolecule of defined size. No significant inhibitory activity could be demonstrated after gel filtration of a DNA-cellulose flow-through of DOC-treated cytosol from TPA-stimulated 70Z/3 cells (*20*), implying that TPA treatment of cells inactivated IκB.

The inhibitor fraction was treated with trypsin to test whether IκB is a protein (Fig. 2B). Tryptic digestion was stopped by the addition of bovine pancreas trypsin inhibitor (BPTI), and samples were analyzed for NF-κB inhibition. Trypsin treatment interfered with the activity of IκB, as shown by the complete inability of the treated sample to inhibit NF-κB activity (Fig. 2B, compare lanes 1 and 6). Trypsin that had been treated with BPTI had no effect (Fig. 2B, lane 5) demonstrating that the inactivation of IκB was specifically caused by the proteolytic activity of trypsin. It appears that IκB requires an intact polypeptide structure for its activity.

The cytosolic complex of IκB and NF-κB showed an apparent size of about 120 to 130 kD after both gel filtration (Fig. 2A, lane 3) and sedimentation through a glycerol gradient (Fig. 2C, lanes 6 and 7). For both methods, cytosol from unstimulated cells was analyzed under nondissociating conditions. The NF-κB was activated in fractions by either DOC (Fig. 2A) or formamide (*19*) (Fig. 2C, middle panel) prior to analysis by EMSA. The specificity of complexes was tested with a mutant DNA probe (*9*) (Fig. 2C, right panels). The apparent release of a 60- to 70-kD inhibitory protein from the cytosolic NF-κB precursor, its sedimentation velocity in glycerol gradients, and its size seen by gel filtration suggest that the inactive NF-κB precursor is a heterodimer composed of a 55- to 62-kD NF-κB molecule (*19*) and a 60- to 70-kD IκB molecule. Nuclear NF-κB was found to cosediment with the cytosolic complex of IκB and NF-κB (Fig. 2C, upper panel). Native gel electrophoresis, a method that allows resolution of size differences of protein-DNA complexes (*21*), provided evidence that the 120 kD form of nuclear NF-κB seen in glycerol gradients comes from the formation of a homodimer (*22*). By these interpretations, activation of NF-κB would include an additional step, that is, formation of a NF-κB homodimer. This is consistent with the observation that the protein-DNA complexes formed with in vitro–activated NF-κB have the same mobility in native gels as those formed with nuclear NF-κB (*19*).

Inactivation of NF-κB by IκB is reversible, saturable, and specific. Incubation with the inhibitor fraction can inhibit the DNA binding activity of NF-κB by more than 90 percent (Fig. 3A, lanes 1 and 3). Treatment of a duplicate sample with DOC after the inhibition reaction reactivated 66 percent of the added NF-κB activity (Fig. 3A; compare lanes 3, 4, and 6). This showed that a DOC-dependent form of NF-κB can be reconstituted in vitro by the addition of a fraction containing IκB to nuclear NF-κB. The incomplete activation of NF-κB by DOC might be due to the DOC-

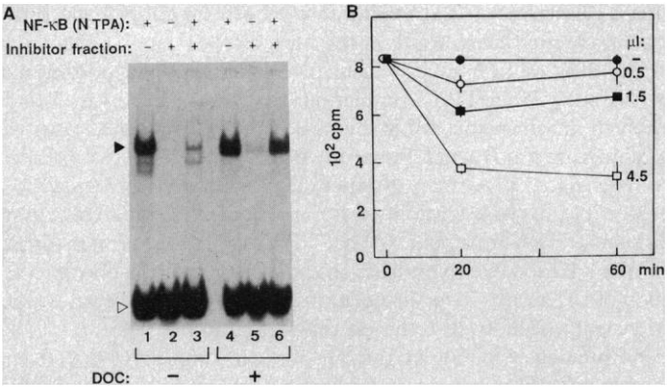

Fig. 3. Reversibility and kinetics of the inactivation of NF-κB. (**A**) The effect of DOC treatment on in vitro inactivated NF-κB. The NF-κB contained in nuclear extracts from TPA-stimulated 70Z/3 cells (N TPA; 1.1 μg of protein) was inactivated by addition of a gel filtration fraction containing IκB (2.5 μg of protein). A duplicate sample was treated after the inhibition reaction with 0.8 percent DOC followed by addition of DNA binding reaction mixture (*43*) containing 0.7 percent NP-40. Samples were analyzed by EMSA. In the fluorograms shown, the filled arrowhead indicates the position of the NF-κB–DNA complex, and the open arrowhead the position of unbound DNA probe. (**B**) A titration and kinetic analysis of the in vitro inactivation of NF-κB. The NF-κB contained in nuclear extracts from TPA-treated 70Z/3 cells (2.2 μg of protein) was incubated with increasing amounts (0.25 to 2.25 μg of protein) of a gel filtration fraction containing IκB. After the DNA binding reaction, samples were analyzed by EMSA. The ^{32}P radioactivity in the NF-κB–DNA complexes visualized by fluorography was determined by liquid scintillation counting. All reactions were performed in triplicates. The bars represent standard deviations.

neutralizing effect of non-ionic detergent that was still present in the sample from the preceding inhibition reaction.

A titration and kinetic analysis showed that IκB stoichiometrically interacts with NF-κB (Fig. 3B). Increasing amounts of inhibitor fraction were added to an excess amount of NF-κB and incubated for 20 or 60 minutes. After the DNA binding reaction, NF-κB-DNA complexes were separated on native gels and quantified by liquid scintillation counting. The relation between amount of IκB fraction added and extent of inhibition was linear. The amount of NF-κB inactivated after 20 minutes of incubation was not increased after 60 minutes (Fig. 3B). These kinetics were probably not the result of a rapid decay of a catalytically active inhibitor because the fractions were incubated before the reaction. Our data are consistent with rapid formation of an inactive complex by addition of IκB to NF-κB. The fraction containing IκB does not appear to catalytically or covalently inactivate NF-κB: neither the reversibility nor the kinetics support such a model.

IκB was tested for its influence on the DNA binding activity of other defined nuclear factors (Fig. 4A). These factors were contained in nuclear extracts that had essentially no active NF-κB (*19*), which otherwise could have inactivated IκB by complex formation. The DNA binding activity of H2TF1, a transcription factor thought to be related to NF-κB (*23*) was not affected by the inhibitor fraction. Ubiquitous and lymphoid-specific octamer-binding proteins (OCTA) (*24, 25*) were unaffected in their DNA binding activities as were two E-box binding factors, NF-μE1 (*26*) and NF-κE2 (*9*), interacting with μ heavy chain and κ light chain enhancers, respectively. Another TPA-inducible transcription factor, AP-1 (*13*), also showed equal complex formation after incubation in the presence and absence of the inhibitor fraction. Furthermore, none of the undefined DNA binding activities seen in the EMSA showed any inactivation by IκB (Fig. 4A). These results show that IκB is a specific inhibitor of the DNA binding activity of NF-κB.

In vivo activated NF-κB is responsive to IκB. IκB prepared from the mouse pre-B cell line 70Z/3 was tested for inactivation of NF-κB contained in nuclear extracts from other cell lines. Human NF-κB contained in nuclear extracts from TPA-stimulated HeLa cells and H-9 T lymphoma cells was efficiently inactivated (Fig. 4B). When excess amounts of the various NF-κB activities were used in the inhibitor assay, the extent of reduction of NF-κB activities by a fixed amount of IκB was very similar as determined by liquid scintillation counting (*20*). Also NF-κB from nuclear extracts of TPA-stimulated Madin-Darby bovine kidney (MDBK) cells was inactivated (*20*), suggesting that the control of NF-κB activity by IκB is conserved among different mammalian species.

NF-κB is constitutively active in cell lines derived from mature B cells (*18*). We tested nuclear extracts from the mouse B cell line WEHI 231 in the inhibitor assay to examine whether NF-κB has undergone a modification in those cell lines that prevented its inactivation by IκB. The NF-κB from B cells was as efficiently inactivated as NF-κB from pre-B cells (Fig. 4B), suggesting that NF-κB is not stably modified in B cells (or in other cells after TPA stimulation) in such a way that it cannot respond to inactivation by IκB.

The NF-κB–IκB complex is present in enucleated cells. The NF-κB–IκB complex shows a cytosolic localization on subcellular fractionation (*19*). This procedure may, however, cause artifacts. Hypotonic lysis of cells may result in partitioning of nuclear proteins into the cytosol, especially when they are not tightly associated with nuclear components (*27*). We tried therefore to detect the complex of IκB and NF-κB in enucleated cells. Enucleation is performed with living cells at 37°C (*28*) and should therefore not interfere with active nuclear import of proteins, which is adenosine triphosphate–dependent and blocked at low temperature (*29*).

Using cytochalasin B–treated HeLa cells, we obtained an enucleation efficiency of about 90 percent (Fig. 5A). Enucleated and cytochalasin B–treated complete cells were incubated in the absence and presence of TPA and solubilized by detergent; and the proteins were extracted with high salt. Because of the small number of cells analyzed, this procedure is different from our standard one. Total cell extracts were analyzed for NF-κB–specific DNA binding activity by EMSA (Fig. 5B). In both enucleated and complete cells, similar amounts of NF-κB activity were found after TPA stimulation (Fig. 5B, lanes 1 to 4). The activity was specific for NF-κB because it was not observed with a mutant κ enhancer fragment (*9, 20*). These results suggest that TPA-inducible NF-κB in HeLa cells is predominantly cytoplasmic because it was still present in enucleated cells. The NF-κB activity seen under control conditions (Fig. 5B, lanes 1 and 3) was most likely activated by the lysis conditions used because the activity was also observed in extracts from HeLa cells that were not treated with cytochalasin B (*20*) but not in fractions obtained after hypotonic lysis (*19*). It was still evident, however, that TPA could activate NF-κB in enucleated cells (Fig. 5B, lanes 3 and 4).

After treatment with DOC, total extracts from complete and enucleated control cells showed about a twofold increase in the amount of NF-κB activity (Fig. 5B, compare lanes 1 and 3 with 5 and 7). The demonstration of DOC-activatable NF-κB in enucleated cells as well as the presence of similar amounts of total NF-κB in enucleated and complete cells (Fig. 5B, lanes 5 to 8) shows that a substantial amount of the total cellular NF-κB–IκB complex was cytoplasmic. In contrast to NF-κB, most of the DNA binding activity of AP-1, a bona fide nuclear protein (*13*), was apparently lost by enucleation of cells (Fig. 5B, lanes 9 to 12).

Mechanism of NF-κB activation. We have shown that the NF-κB nuclear transcription factor exists in unstimulated pre-B cells in a cytoplasmic complex with a specific inhibitory protein, IκB. In this complex, NF-κB does not exhibit DNA binding activity in EMSA

and partitions, upon subcellular fractionation, into the cytosol. The complex is apparently a heterodimer consisting of an NF-κB molecule of about 60 kD and an IκB molecule of 60 to 70 kD. Upon TPA stimulation of cells or after treatment with dissociating agents in vitro, the NF-κB–IκB complex dissociates. This releases NF-κB, which appears now to form a homodimer and can translocate into the nucleus. Whether dimerization is required for activation of NF-κB is not known.

The inhibitory effect of IκB on the DNA binding activity and nuclear localization properties of NF-κB appears to arise from a simple physical affinity of the two proteins. The complex freely dissociates and the components readily associate under in vitro conditions. Even in vivo, dissociation by short-term TPA treatment and reassociation after long-term TPA treatment (*30*) is evident. The latter presumably results from the degradation of protein kinase C after TPA activation and implies that NF-κB can move back to the cytoplasm after being active in the nucleus.

The effect of TPA appears to involve an alteration of IκB but not of NF-κB. After TPA stimulation, no active IκB was found, implying its alteration, while the nuclear NF-κB remained sensitive to unmodified IκB when tested in vitro. Whether inactive IκB can be regenerated is unclear; in experiments with cycloheximide (*30*), irreversible loss of IκB activity was the only demonstrable effect after 8 hours of TPA treatment. In that TPA can activate protein kinase C, it is a reasonable hypothesis that direct or indirect phosphorylation of IκB results in its dissociation from NF-κB; however, we are at present unable to directly test this suggestion.

We previously found that the NF-κB–IκB complex is recovered in the cytosol (*19*). Here we show directly that it is not removed from the cell by enucleation and therefore is truly cytoplasmic (*31*). Because active protein kinase C is bound to the plasma membrane (*32*), it becomes increasingly attractive to suggest that the cytoplas-

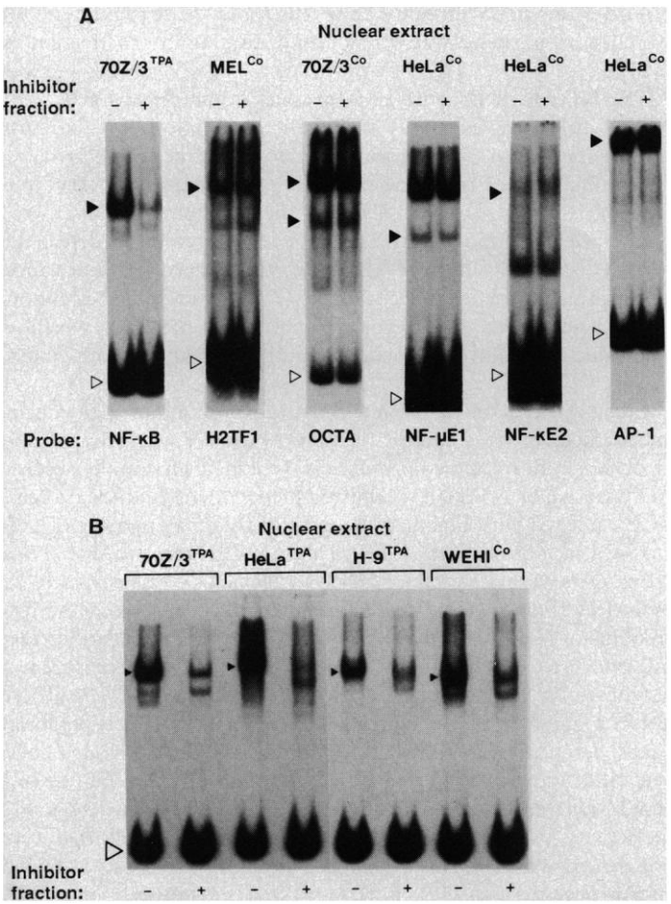

Fig. 4. Specificity of IκB. Nuclear extracts from unstimulated (Co) or TPA-treated cells were incubated with 5 μl of buffer G (−) (*41*) or with 5 μl of a gel filtration fraction containing IκB (+) (A, in the presence of 150 m*M* NaCl). After DNA binding reactions, samples were analyzed by EMSA. (**A**) Influence of IκB on the DNA binding activity of various nuclear factors. The probes were: NF-κB (*18*); H2TF1, an oligonucleotide subcloned into pUC containing the H2TF1 binding site from the H-2 promoter (*23*); OCTA, an oligonucleotide subcloned into pUC containing the common binding site for the ubiquitous (upper filled arrowhead) and lymphoid-specific (lower filled arrowhead) octamer-binding proteins (*25*); NF-μE1 (*26*); NF-κE2 (*9*); and AP-1, Eco RI–Hind III fragment of the yeast HIS4 promoter (*47*) containing three binding sites recognized by mammalian AP-1/jun (*48*). In the fluorograms shown, filled arrowheads indicate the positions of specific protein-DNA complexes. Open arrowheads indicate the positions of uncomplexed DNA fragments. (**B**) Interaction of IκB with NF-κB from different cell lines. The filled arrowheads indicate the positions of the NF-κB-DNA complexes from the various cell lines and the open arrowhead indicates the position of uncomplexed DNA probe.

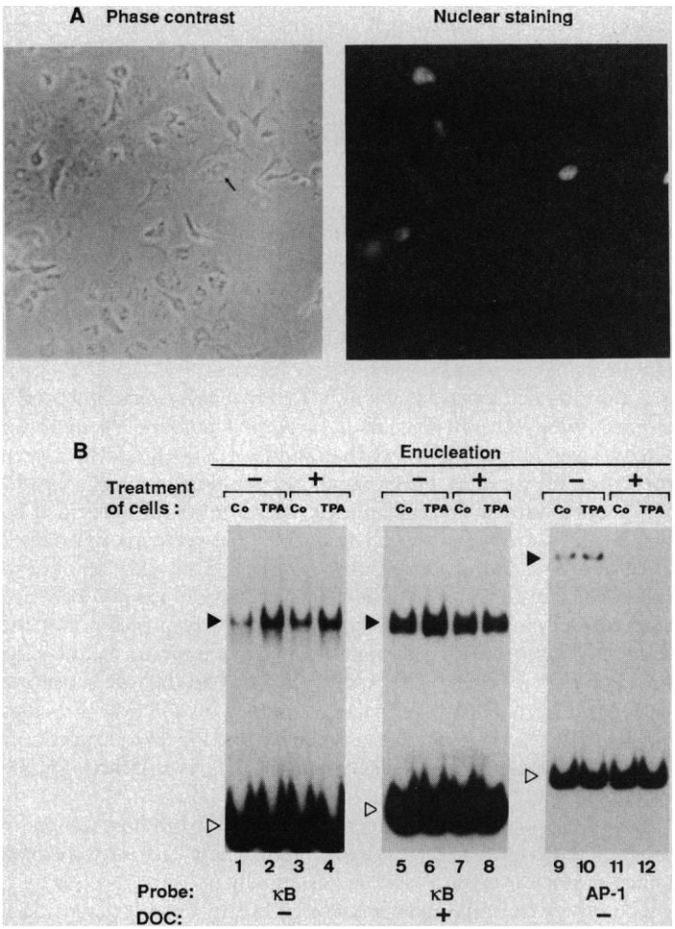

Fig. 5. Presence of NF-κB in enucleated cells. (**A**) Phase contrast and fluorescence microscopy of enucleated HeLa cells (*49*). From 612 cells counted on photographic prints, 63 showed nuclear staining. A representative micrograph is shown. The arrow indicates a cell that retained its nucleus. (**B**) Analysis of complete and enucleated cells for NF-κB activity. Total cell extracts (1.2 μg of protein) from control (Co) and TPA-treated complete and enucleated cells were analyzed by EMSA with a labeled κ enhancer fragment (κB) or HIS4 promoter fragment (AP-1) (*47, 48*), 3 μg of poly(dI-dC), 1 μg of BSA, 1.2 percent NP-40 and the binding buffer (*19*) in a final volume of 20 μl. In lanes 5 to 8, extracts were treated with DOC followed by the addition of the DNA binding mixture to give final concentrations of 0.8 percent DOC and 1.2 percent NP-40. Samples were analyzed by EMSA. In the fluorograms shown, the filled arrowheads indicate the positions of specific protein-DNA complexes and the open arrowheads indicate the positions of uncomplexed DNA probes.

mic complex interacts in the cytoplasm (perhaps near the plasma membrane) with protein kinase C, and the liberated NF-κB carries the signal from cytoplasm to nucleus. Under a number of conditions, active NF-κB is found in the cytoplasm (*30*): this and the reversibility of NF-κB activation in vivo (*30*) suggests that the protein may freely move in and out of the nucleus, bringing to the nucleus information reflecting the cytoplasmic activation state of protein kinase C and possibly of other signaling systems.

The response of NF-κB to activated protein kinase C occurs apparently indirectly through modification and subsequent release of associated IκB. The inducibility of NF-κB by TPA is thus dependent on the presence and state of activity of IκB. Changes in amount or activity of IκB should therefore influence the TPA inducibility of NF-κB. The NF-κB can indeed exist not only in TPA-inducible but also in constitutively active form, for example, in mature B cells (*18*). Because constitutive NF-κB from B cells is still responsive to IκB in vitro, we suspect that IκB and not NF-κB is altered during differentiation of pre-B into B cells.

IκB is apparently unstable when not complexed with NF-κB. This is suggested by the absence of excess active inhibitor in the cytosol from unstimulated cells. In a situation where the production of new inhibitor is impaired, the decay of occasionally released inhibitor could activate NF-κB. This would explain the partial activation of NF-κB seen after treatment with the protein synthesis inhibitors cycloheximide and anisomycin (*11*). The demonstration of a specific inhibitory protein of NF-κB and the interpretation that cycloheximide treatment can activate NF-κB presumably because cells become depleted of inhibitor suggest that IκB is the putative labile repressor of κ gene expression (*33*) and of NF-κB activity (*11*).

Similarity to the activation of glucocorticoid receptor. Inactive glucocorticoid receptor (GR) is localized in the cytosol (*34*) in complex with two molecules of the 90-kD heat shock protein (hsp90) (*35*). This complex is thought to dissociate upon binding of steroid hormone to the receptor (*36, 37*), thereby releasing active GR that is able to migrate into the nucleus to bind to glucocorticoid responsive elements (*38*). Despite a similar function, we consider it unlikely that IκB is related to hsp90. The molecular size, specificity, responsiveness to TPA, and apparent low abundance and lability distinguish IκB from hsp90. Unlike the GR-hsp90 complex (*36*), the NF-κB–IκB complex is stable to warming, gel filtration, dilution, and elevated ionic strength (*19*, and our data presented above) suggesting a high affinity interaction between IκB and NF-κB. In the case of the GR-hsp90 complex, there is no requirement for a high affinity interaction because hsp90, as a very abundant protein (*39*), is in great excess over the receptor. Furthermore, modification of hsp90 is apparently not required for dissociation of the complex. It is, rather, released by a conformational change of the receptor upon hormone binding (*37*).

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41. Cytosol from unstimulated 70Z/3 pre-B cells in buffer A (*19, 44*) was adjusted to a final concentration of 50 m*M* NaCl, 20 m*M* Hepes (*p*H 7.9), 1.5 m*M* EDTA, 5 percent glycerol and 0.2 percent NP-40. Cytosolic protein (45 mg) was mixed to a final volume of 4 ml with 0.6 percent DOC, 0.75 g of calf thymus (wet weight) DNA-cellulose [Sigma; equilibrated in buffer G: 10 m*M* tris-HCl (*p*H 7.5), 50 m*M* NaCl, 1 m*M* EDTA, 1 m*M* dithiothreitol (DTT), 5 percent glycerol, 0.2 percent DOC, 0.2 percent NP-40, and 0.5 m*M* phenylmethyl sulfonylfluoride (PMSF)] and 1.2% NP-40. The suspension was incubated in a mini column for 1 hour at room temperature on a rotary shaker. The flow-through fraction was used for gel filtration. DNA-cellulose was washed with buffer G and eluted with a NaCl step gradient in buffer G. Equal proportions of fractions were assayed by EMSA (*18, 19*) at a final concentration of 1.2 percent NP-40 in the presence of either 0.03 percent DOC (nondissociating condition) or 0.6 percent DOC (dissociating condition) and with 10 μg of bovine serum albumin (BSA) as carrier.
42. The flow-through fraction from the DNA-cellulose column (1.55 mg of protein in 250 μl) was subjected to a G-200 Sephadex column (280 by 7 mm) with a flow rate of 0.15 ml/min in buffer G at room temperature. A mixture of size markers (dextran blue; immunoglobulin G, 158 kD; BSA, 67 kD; ovalbumin, 45 kD; myoglobin, 17 kD; Bio-Rad) was run on the column before samples were analyzed. Markers were detected in fractions by their color and by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), which was followed by Coomassie blue staining.
43. To detect inhibiting activity, portions of fractions (5 μl; in buffer G) were mixed with 1 μl of nuclear extracts [in buffer D(+) (*19, 44*)] and 0.5 μl 10 percent NP-40. After 30 minutes at room temperature, the reaction volume was brought to 20 μl by the addition of a DNA binding reaction mixture containing 3.2 μg of poly(dI-dC) (Pharmacia), 1 to 4 fmole of ^{32}P–end labeled κ enhancer fragment (*18*), 75 m*M* NaCl, 15 m*M* tris-HCl (*p*H 7.5), 1.5 m*M* EDTA, 1.5 m*M* DTT, 7.5 percent glycerol, 0.3 percent NP-40, and 20 μg of BSA. After a 20-minute DNA binding reaction, samples were analyzed by EMSA (*18, 19*).
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46. Nuclear extract from TPA-stimulated 70Z/3 cells and cytosol from untreated cells (both 220 μg of protein) were sedimented through 5 ml of a continuous 10 to 30 percent glycerol gradient in buffer D(+) at 150,000*g* (SW 50.1 rotor; Beckman) for 20 hours at 4°C. Cosedimented size markers were detected in fractions by SDS-PAGE and Coomassie blue staining. Portions of glycerol gradient fractions (4 μl) were analyzed by EMSA (*19*) with 10 μg of BSA as carrier and 0.5 μg of poly(dI-

dC). The NF-κB precursor was activated by treating 4 μl of fractions with 1.5 μl of formamide before the DNA binding reaction mixture was added.

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49. HeLa cells were grown in Eagle's minimum essential medium supplemented with 10 percent horse serum, penicillin (50 IU/ml), and streptomycin (50 μg/ml) (referred to as supplemented MEM) on disks (1.8 cm in diameter) cut from cell culture plastic ware. For enucleation, disks were placed upside down into centrifuge tubes filled with 10 ml of supplemented MEM at 37°C containing cytochalasin B (10 μg/ml; Sigma). Cells were enucleated for 20 minutes by centrifugation at 17,500*g* (JS-13 rotor; Beckman) at 37°C. Control cells were also treated with cytochalasin B (10 μg/ml) and held for the same time in the incubator. To estimate the enucleation efficiency, enucleated cells on one disk were fixed with formaldehyde (3.7 percent) in phosphate-buffered saline (PBS) for 20 minutes, stained for 4 minutes with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml; Sigma) in PBS, and washed in PBS. Fluorescence microscopy under ultraviolet and phase contrast microscopy were performed with a Zeiss Photomicroscope III. Control and enucleated cells were allowed to recover in cytochalasin B—free supplemented MEM for 30 minutes before a 2-hour incubation in the absence or presence of TPA (50 ng/ml). Cells were then washed in ice-cold PBS, scraped off the disks in 100 μl of a buffer containing 20 m*M* Hepes (*p*H 7.9), 0.35*M* NaCl, 20 percent glycerol, 1 percent NP-40, 1 m*M* $MgCl_2$, 1 m*M* DTT, 0.5 m*M* EDTA, 0.1 m*M* EGTA, 1 percent aprotinin (Sigma) and 1 m*M* PMSF. After lysis and extraction for 10 minutes on ice, particulate material was removed by centrifugation (Microfuge) for 15 minutes at 4°C, and the resulting supernatants were analyzed by EMSA.
50. We thank K. Arndt, A. Baldwin, J. LeBowitz, and C. Murre for providing labeled DNA probes to detect various DNA binding activities and for a nuclear extract from MEL cells; M. Lenardo for plasmids containing wild-type and mutant κ enhancer and for a nuclear extract from MDBK cells; R. Van Etten and T. Orr-Weaver for help with fluorescence microscopy; L. Staudt for stimulating discussion; M. Smith for carefully reading the manuscript; and Owl Scientific (Cambridge, MA) for preparation of disks from cell culture plastic ware. Supported by a grant from the American Cancer Society (D.B.) and a fellowship of the Deutsche Forschungsgemeinschaft (P.A.B.).

27 July 1988; accepted 26 September 1988