

Cytoskeletal Control of Gene Expression: Depolymerization of Microtubules Activates NF- κ B

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Abstract. Cell shape changes exert specific effects on gene expression. It has been speculated that the cytoskeleton is responsible for converting changes in the cytoarchitecture to effects on gene transcription. However, the signal transduction pathways responsible for cytoskeletal-nuclear communication remained unknown. We now provide evidence that a variety of agents and conditions that depolymerize microtubules activate the sequence-specific transcription factor NF- κ B and induce NF- κ B-dependent gene expression. These effects are caused by depolymerization of microtubule because they are blocked by the microtubule-stabilizing agent taxol. In nonstimulated cells, the

majority of NF- κ B resides in the cytoplasm as a complex with its inhibitor I κ B. Upon cell stimulation, NF- κ B translocates to the nucleus with concomitant degradation of I κ B. We show that cold-induced depolymerization of microtubules also leads to I κ B degradation and activation of NF- κ B. However, the activated factor remains in the cytoplasm and translocates to the nucleus only upon warming to 37°C, thus revealing two distinct steps in NF- κ B activation. These findings establish a new role for NF- κ B in sensing changes in the state of the cytoskeleton and converting them to changes in gene activity.

A variety of observations suggest that changes in cell shape or architecture can regulate gene expression (6, 35). The shape of a cell is affected by interactions with either the extracellular matrix or neighboring cells, which lead to restructuring of the cytoplasmic cytoskeleton. Altering cytoskeletal structure may in turn change the availability of regulatory or catalytic sites of key signal-transducing molecules. Some of the classical examples illustrating the effect of cell shape changes on nuclear gene expression include a switch from type I to type II collagen expression by chondrocytes upon a shift from growth on fibronectin to growth in suspension (7), as well as the repression of liver-specific gene expression in dispersed hepatocytes and its resumption upon cell aggregation (14). The exact mechanisms by which changes in cell shape are converted to changes in the pattern of gene expression have not been explained, but the cytoskeleton is a good candidate to have a key role in this mysterious signal transduction process. It has been suggested that the cytoskeleton may regulate gene expression by interacting with the nuclear matrix (9), and that this interaction may lead to physical expansion of nuclear pores and thereby increase the rate of nuclear transport in spreading cells (35).

One of the major components of the cytoskeleton is the microtubule network. Because of the dynamic instability of tubulin dimers, this structure is subject to constant remodel-

ing. Massive microtubule reorganization occurs in the mitotic phase of the cell cycle (46), during cell differentiation (36), upon specific binding of cytotoxic T lymphocytes to its target cell (24), and during injury-induced migration of endothelial cells to wounded tissue (27). This massive microtubule reorganization correlates with changes in gene expression. Depolymerization of microtubules with drugs can stimulate cell proliferation in the absence of other signals (15), whereas stabilization of microtubules can inhibit the action of certain mitogens (16). It was shown that transcription of several genes, including the ones that encode urokinase type plasminogen activator (uPA)¹ (11, 38) and interleukin IL-1 β (23, 44) is activated by microtubule-depolymerizing agents such as nocodazole and colchicine.

One plausible way by which the cytoplasmic cytoskeleton can affect nuclear gene expression is by modulating the activity of transcription factors that reside in the cytoplasm of unstimulated cells in an inactive form and migrate to the nucleus in response to various stimuli (34). Such factors are the NF- κ Bs, a collection of structurally and functionally related dimeric complexes that activate transcription of immunoregulatory genes that encode cell surface receptors, cytokines, acute phase reactants, and cell adhesion molecules (reviewed in reference 28). NF- κ B was originally characterized as a heterodimer of two subunits, p50 and p65, each of which is also capable of homodimerizing and binding

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1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; TPA, tetradecanoyl phorbol acetate; uPA, urokinase plasminogen activator.

DNA on its own (25, 37, 50, 55, 56). The NF- κ B family now consists of multiple dimer-forming proteins with homology to the *rel* oncogene (28). The cellular localization, DNA binding, and transcriptional properties of the NF- κ B proteins are regulated by a second family of proteins, the I κ Bs, that contain ankyrin-type repeats (10, 26, 41). The ankyrin-type repeats of ankyrins are thought to form binding sites for integral membrane proteins and tubulin (17, 43). It was proposed that I κ B prevents NF- κ B nuclear translocation by masking the nuclear localization signal located in the Rel homology domain common to these proteins (5, 62). This interaction is mediated through the ankyrin-type repeats of I κ B (31). However, it is also possible that the ankyrin-type repeats of I κ B may make additional contribution to the cytoplasmic retention of NF- κ B through an interaction with cytoskeletal components. This may allow the activity state of NF- κ B to be modulated by the cytoarchitecture and provide a mechanism for gene regulation in response to cytoskeletal changes. In this paper, we demonstrate that microtubule depolymerization can lead to activation of NF- κ B- and NF- κ B-dependent gene expression. These findings establish a signal transduction pathway that interprets changes in the state of the cytoplasmic cytoskeleton and converts them to changes in the activity of a sequence-specific transcription factor.

Materials and Methods

Reagents

TPA (stored at 100 ng/ μ l in ethanol), nocodazole (4 mg/ml in DMSO), colchicine (0.4 mg/ml in DMSO), vinblastine (10 mg/ml in ethanol), podophyllotoxin (50 μ g/ml in PBS), taxol (10 mM in DMSO), β -lucicolchicine (0.4 mg/ml in DMSO), cytochalasin D (1 mg/ml in DMSO), cycloheximide (10 μ g in PBS), and monoclonal anti- β -tubulin (mAb TUB2.1) were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-p50 antibody was generated against a synthetic NH₂-terminal peptide (MAEDDPYLGRPEQK), rabbit anti-p65 antibody was generated against recombinant p65 (45), and rabbit anti-I κ B α was generated against recombinant I κ B α (21).

Cell Culture and Transfection

HeLa S3 cells were maintained in DME supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. For induction experiments, cells were incubated with DME containing 0.5% FBS for 24 h and then treated with the different agents for the indicated time periods. Human peripheral blood mononuclear cells prepared from whole heparinized blood by density-gradient centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) were enriched in monocytes by adherence to plastic tissue culture plates for 4 h at 37°C in RPMI containing 3% human serum. Nonadherent cells were removed by washing with media and then treated with the different agents. For transfection experiments, cells were fed with fresh medium containing 10% FBS 3 h before incubation with calcium phosphate-DNA precipitate containing 2 \times I κ B-CAT (5 μ g), 2 \times mI κ B-CAT (5 μ g), or -73 Col-CAT (2 μ g). Each 100-mm culture dish was transfected with a total DNA amount adjusted to 10 μ g with pUC18. Precipitates were left on cells for 4 h before shocking with 10% glycerol for 3 min. Cells were washed twice with PBS and then refed with DME containing 0.5% FBS. Treatments with tetradecanoyl phorbol acetate (TPA) (100 ng/ml) or nocodazole (0.4 μ g/ml) were initiated 24 h later. The cells were harvested after a 12-h induction period. Extracts were prepared by sonicating, and chloramphenicol acetyl transferase (CAT) enzyme activity was determined as described (2).

Preparation of Cell Extracts, Mobility Shift Assays, and Immunoblotting

Cells were washed twice with PBS and then scraped off the plate with a rubber policeman. For preparation of whole cell extracts, cell pellets were resuspended in high salt buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl,

1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 2.5 μ g/ml aprotinin, 2.5 μ g/ml leupeptin, 1 μ g/ml bestatin, and 1 μ g/ml pepstatin) containing 0.02% NP-40 and rotated at 4°C for 30 min. Particulate material was removed by centrifugation at 10,000 g for 5 min. For preparation of cytoplasmic and nuclear extracts, cell pellets were resuspended in hypotonic buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitors as indicated above), and they were allowed to swell on ice for 15 min. NP-40 was added to 0.02%, and the suspension was passed six times through a 26.5-gauge needle. Nuclei were pelleted for 1 min at 10,000 g, and the supernatant was recovered as the cytoplasmic extract. The nuclear pellet was resuspended in buffer C and rotated at 4°C for 30 min. Particulate material was pelleted, and the supernatant was recovered as described above. For the mobility shift assays, protein-DNA complexes were formed at 4°C for 30 min in 20 or 30 μ l of 12 mM Hepes, pH 7.9, 4 mM Tris-HCl, pH 7.9, 60 mM KCl, 30 mM NaCl, 5 mM MgCl₂, 5 mM DTT, 0.1 mM EDTA, 12.5% glycerol containing 5 μ g poly(dI-dC), and 0.05 ng of ³²P-labeled DNA probe (~10,000 cpm). The oligonucleotide sequences are as follows:

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PD      5' -TCGAGCGGCAGGGGAATTCCTCTCC- 3'
        3' -CGCGTCCCTTAAGGGGAGAGGAGCT- 5'

Col-TRE 5' -AGCTTAAGCATGAGTCAGACACCT- 3'
        3' -ATTTCTACTCAGTCTGTGGACTTAA- 5'

Sp-1    5' -CGATCGGGGCGGGGCGCATCGGGGCGGGGCG- 3'
        3' -GCTAGCCCCGCCCGCGCTAGCCCCGCCCGC- 5'

 $\kappa$ B-IL1 $\beta$  5' -AGCTTGAGACTCATGGGAAATCCACATTTGAT- 3'
        3' -CTCTGAGTACCCTTTTAGGGTGAAACTATTCGA- 5'

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To determine the binding specificity and nature of the complexes, cold oligonucleotides or antibodies were preincubated with the extracts for 30 min at 4°C before incubation with the reaction cocktail containing the probe. Protein-DNA complexes were resolved on 5% or 6% nondenaturing polyacrylamide gels (acrylamide/bisacrylamide ratio = 29:1) containing 0.25 \times TBE buffer at 200 V for 2 h at room temperature.

To detect I κ B α , 10–20 μ g cytoplasmic or whole-cell extracts were separated on 12% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) for 3 h in buffer containing 25 mM Tris base, 200 mM glycine, and 15% methanol, then blocked in 5% dry nonfat milk in PBS for 2–12 h, and then incubated in rabbit polyclonal antibody to I κ B α , followed by anti-rabbit IgG coupled to horseradish peroxidase (Amersham Corp., Arlington Heights, IL). Immunoreactive bands were detected with the ECL chemiluminescent kit (Amersham).

Preparation of dimeric and polymeric fractions of tubulin was described as in (40). Briefly, dimeric fractions were collected using lysis buffer containing 80 mM Pipes-KOH, pH 6.8, 1 mM MgCl₂, 2 mM EGTA, 0.5% Triton X-100, and polymeric fractions were extracted from the remaining Triton-insoluble material with 1% SDS in water. Extracts were analyzed by immunoblotting with anti- β -tubulin, and immunoreactive bands were detected as above. Chemiluminescence from each band was quantitated after phosphorimaging in a molecular imaging system (GS-250; Bio Rad Laboratories, Hercules, CA).

Results

Activation of NF- κ B by Nocodazole

To investigate whether disruption of the microtubule network affects NF- κ B activity, we treated serum-starved HeLa S3 cells with nocodazole, a reversible inhibitor of tubulin polymerization (18). Nuclear extracts were examined for NF- κ B-binding activity by a mobility shift assay, using a palindromic NF- κ B-binding site (60). As shown in Fig. 1 *a*, nocodazole treatment resulted in rapid activation of NF- κ B-binding activity. The time course of NF- κ B activation by nocodazole lagged the time course of microtubule depolymerization by 15 min, determined either by staining with anti- β -tubulin antibody in indirect immunofluorescence of intact cells (data not shown) or by immunoblot analysis of dimeric and polymeric tubulin fractions (Fig. 1 *b*). Although considerable depolymerization of microtubules was detected

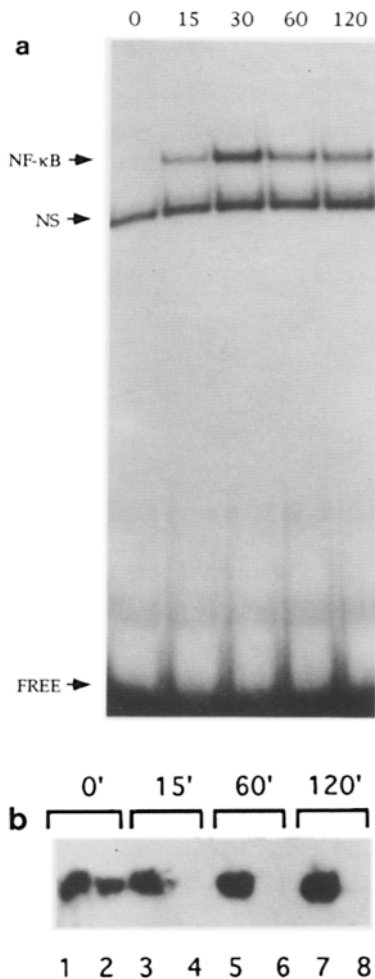


Figure 1. Induction of NF- κ B-binding activity by nocodazole. After serum starvation for 24 h, HeLa S3 cells were treated with 0.4 μ g/ml nocodazole for 15–120 min before harvesting and preparation of whole-cell extracts or Triton-soluble and -insoluble fractions. (a) 5- μ g nuclear extracts were used in a mobility shift assay with a palindromic NF- κ B-binding site as probe. The migration positions of the NF- κ B protein–DNA complex (NF- κ B), a nonspecific protein–DNA complex (NS), and the unbound probe (FREE) are indicated. (b) Portions of the total Triton-soluble and -insoluble fractions (5% of each) were immunoblotted with antibody to β -tubulin. Lanes 1, 3, 5, and 7 were loaded with the Triton-soluble fraction, which contains dimeric tubulin, and lanes 2, 4, 6, and 8 were loaded with the Triton-insoluble fraction, which contains polymeric tubulin.

within 15 min of exposure to nocodazole, NF- κ B-binding activity is detectable after 15 min, but it is not maximum until 30–60 min. This lag in NF- κ B activation suggests that microtubule disruption does not directly induce nuclear NF- κ B-binding activity, but that an intermediate step is necessary for full activation.

In addition to the p50/p65 NF- κ B heterodimer, the palindromic NF- κ B binding site is recognized by the p50 homodimer and much less efficiently by p65 (60). To investigate the nature of the nocodazole-induced NF- κ B-binding activity, we used anti-p50 and anti-p65 antibodies. Incubation with a polyclonal antiserum raised against an NH₂-terminal p50 peptide (45) resulted in complete supershift of a band that previously (reference 52 and unpublished results) was

determined to correspond to the p50 homodimer–DNA complex (Fig. 2). The total amount of the p50 homodimer–DNA complex was increased approximately threefold by the nocodazole treatment. The anti-p50 antiserum had only a small inhibitory effect on the activity of the major nocodazole-induced DNA-binding species, tentatively assigned as the p50/p65 heterodimer. Treatment of the extracts with a polyclonal antiserum raised against p65 (45) resulted in partial inhibition of NF- κ B-binding activity without any noticeable effect on activity of the p50 homodimer. Although these results do not distinguish whether the major (23-fold induction) nocodazole-induced binding species is p50/p65, Rel/p65, or p65 homodimer, the electrophoretic mobility of the nocodazole induced complex is identical to that of a phorbol ester-(TPA) induced NF- κ B complex (Fig. 3). Previous analysis indicates that the major TPA-inducible binding activity in HeLa cells, to the immunoglobulin kappa enhancer, is a p50/p65 heterodimer (21, 29). In addition, p65 homodimers bind rather inefficiently to the palindromic NF- κ B probe (60). Therefore, the nocodazole-induced binding activity is most likely the p50/p65 heterodimer. Oligonucleotide competition experiments confirm that this activity is sequence specific (Fig. 2). Oligonucleotides containing irrelevant binding sites or mutated NF- κ B-binding sites did not compete (data not shown). The experiment shown in Fig.

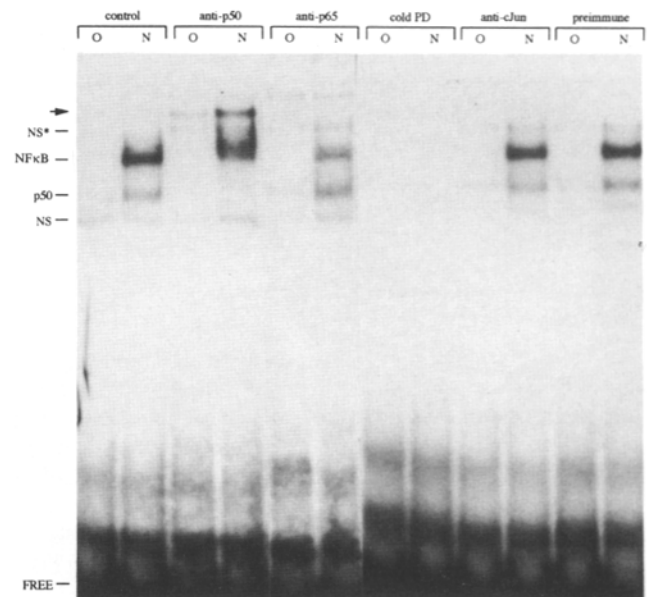


Figure 2. Characterization of the nocodazole-induced NF- κ B-binding activity. Nuclear extracts from untreated (O) or nocodazole- (0.4 μ g/ml; 60 min; N) treated HeLa S3 cells (5- μ g samples) were used in mobility shift assays with the palindromic NF- κ B site as a probe after preincubation with either 2 μ l of rabbit polyclonal antisera raised against either an N-terminal p50 peptide (anti-p50), recombinant p65 (anti-p65), or an NH₂-terminal c-jun peptide (anti-cJun), 2 μ l of preimmune rabbit serum, or no additions (control). The uninduced and nocodazole-induced extracts were also analyzed in the presence of 25-fold molar excess of unlabeled palindromic NF- κ B site (cold PD). The migration positions of the NF- κ B protein–DNA complex (NF- κ B), the p50 homodimer–DNA complex, nonspecific protein–DNA complexes (NS), and the unbound probe (FREE) are indicated. The upper nonspecific complex (NS*) is generated by a component of rabbit serum. The supershifted p50–DNA complex is indicated by the arrow.

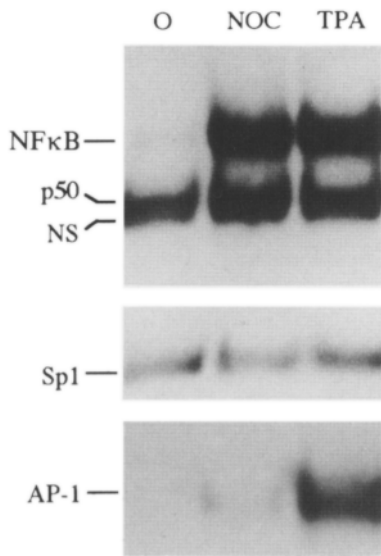


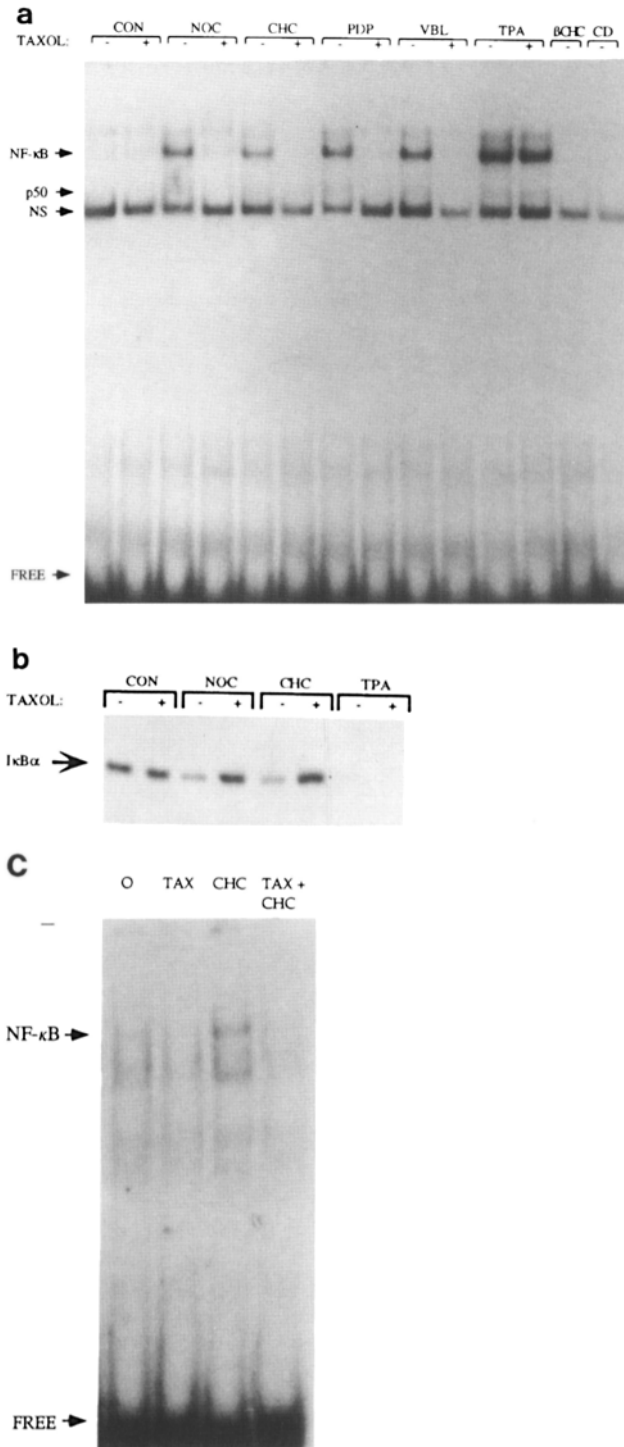
Figure 3. NF- κ B is specifically activated by nocodazole. Nuclear extracts (5 μ g) from HeLa S3 cells that were either nontreated (O) or treated with either nocodazole (0.4 μ g/ml for 60 min; NOC) or TPA (100 ng/ml for 60 min; TPA) were analyzed by mobility shift assays with probes specific for either NF- κ B, Sp1, or AP-1. Only the segments of each gel that contained the protein-DNA complexes are shown in this figure. NS, nonspecific protein-DNA complex.

3 demonstrates that induction of NF- κ B by nocodazole is almost as efficient as its induction by TPA, a well-characterized inducer of NF- κ B activity (39). Induction by nocodazole is specific to NF- κ B since the binding activities of the constitutive transcription factor Sp1 and the TPA-inducible transcription factor AP-1 were not considerably affected by this treatment.

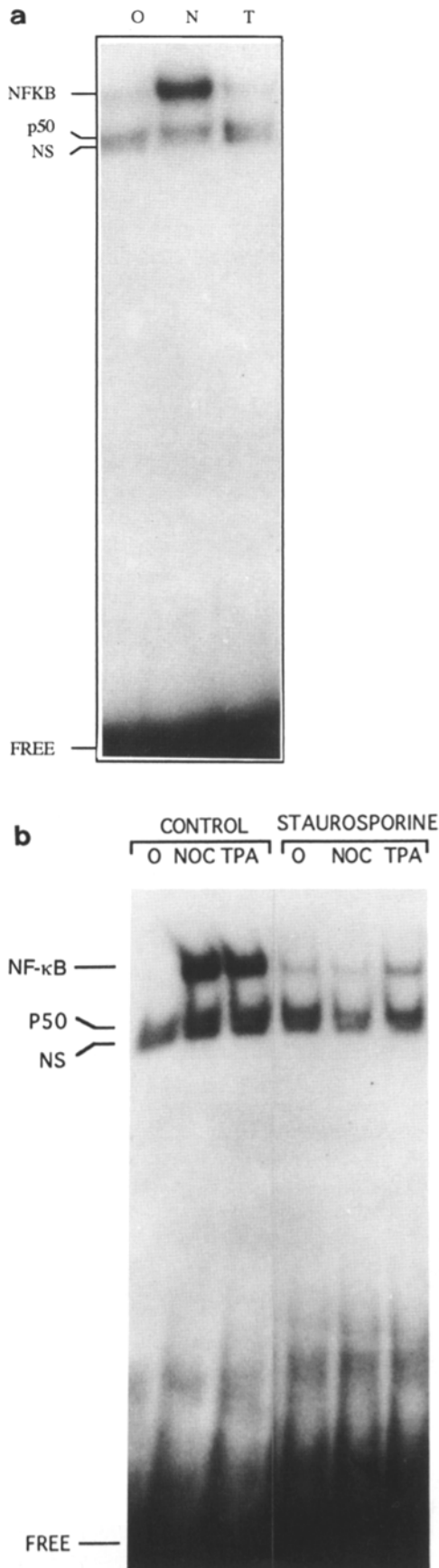
A Variety of Microtubule Disrupting Agents Activate NF- κ B

To determine whether the induction of NF- κ B-binding activity by nocodazole is caused by its antimicrotubule activity, we examined the effect of several microtubule-disrupting agents that have different sites of action on the tubulin dimer (42). As shown in Fig. 4 *a*, treatment with colchicine (CHC), podophyllotoxin (PDP), and vinblastine (VBL) led to rapid and efficient induction of nuclear NF- κ B activity similar to nocodazole, whereas the microtubule-stabilizing agent taxol (20) or the inactive colchicine analog β -lumicolchicine (22) had no effect. An agent that causes depolymerization of actin filaments, cytochalasin D (61), did not lead to induction of nuclear NF- κ B-binding activity. Further proof that the induction of NF- κ B-binding activity is caused by depolymerization of microtubules is provided by the complete inhibition of NF- κ B induction by microtubule-disrupting drugs by pretreatment of the cells with taxol. As indicated by direct

Figure 4. Microtubule depolymerization activates NF- κ B. (a) Serum-starved HeLa S3 cells were preincubated in the absence (-) or in the presence (+) of taxol (5 μ g/ml) for 30 min, followed by an additional 60-min incubation with either nocodazole (NOC; 0.4



μ g/ml), colchicine (CHC; 40 μ g/ml), podophyllotoxin (PDP; 50 ng/ml), vinblastine (VBL; 10 μ g/ml), TPA (100 ng/ml), β -lumicolchicine (β CHC; 40 μ g/ml), cytochalasin D (CD; 2 μ M), or with no further additions (CON). 10 μ g of nuclear extracts were used in a mobility shift assay with the palindromic NF- κ B site as a probe. (b) 10- μ g cytoplasmic extracts from the experiment described above were immunoblotted with anti-I κ B α serum. The specific I κ B α band is indicated. (c) Human peripheral blood monocytes were preincubated in the absence or presence of 5 μ g/ml taxol (TAX) for 30 min, followed by an additional 60-min incubation with 40 μ g/ml colchicine (CHC), or with no further additions (O). 10- μ g whole-cell extracts were used in a mobility shift assay with the IL-1 β NF- κ B site as probe.



examination of tubulin polymerization, taxol pretreatment also prevented microtubule depolymerization induced by these drugs (data not shown). All NF- κ B-inducing agents analyzed thus far, cause the degradation of I κ B (4, 12, 32, 53, 58). As shown in Fig. 4 *b*, induction of NF- κ B-binding activity correlates with the disappearance of I κ B α . The level of I κ B α quantitated by phosphorimage analysis of chemiluminescence from each band was reduced by six- and eight-fold below the control value upon treatment with nocodazole and colchicine, respectively. Taxol, however, had no effect on induction of NF- κ B activity by TPA. NF- κ B activation by microtubule disruption is not restricted to HeLa cells since binding activity to an oligonucleotide corresponding to the NF- κ B-binding site located in the IL-1 β promoter is induced in human peripheral blood monocytes treated with colchicine, and this induction can be blocked with taxol (Fig. 4 *c*).

Although taxol had no effect on induction of NF- κ B activity by TPA, downregulation of protein kinase C by prolonged incubation with TPA prevented the induction of NF- κ B by a second dose of TPA, but had no effect on the response to nocodazole (Fig. 5 *a*). These results suggest that protein kinase C is not required for induction of NF- κ B-binding activity by antimicrotubule drugs and that these agents induce NF- κ B by a different mechanism than TPA. However, a protein kinase is involved since the general protein kinase inhibitor, staurosporin, blocked nocodazole-induced NF- κ B activity (Fig. 5 *b*). As expected, staurosporin also inhibited most of the induction response to TPA.

The effect of nocodazole on microtubule is reversible therefore removal of the drug allows microtubule repolymerization (19). Activation of NF- κ B is reversed within 15 min after removal of the drug unless the protein synthesis inhibitor cycloheximide was present during the recovery period (Fig. 6 *a*, CHX). As indicated by immunoblot analysis of cytoplasmic extracts with anti-I κ B α (Fig. 6 *b*), the return to basal NF- κ B-binding activity correlates with the reappearance of I κ B α . The reappearance of I κ B is most likely caused by new synthesis since it is inhibited by cycloheximide. These results suggest that inhibition of NF- κ B activity after repolymerization of microtubules is dependent on the continuing synthesis of I κ B α . There is a lag in the induction of NF- κ B by microtubule disruption, but its inhibition is rapid after repolymerization.

Figure 5. Effect of downregulation of protein kinase C and a protein kinase inhibitor on activation of NF- κ B by nocodazole. (a) Serum-starved HeLa S3 cells were treated with 100 ng/ml TPA for 24 h and then washed twice with PBS to remove TPA. The cells were then incubated for an additional 60 min in fresh medium alone (O), or in the presence of 0.4 μ g/ml nocodazole (N), or 100 ng/ml TPA (T). Nuclear extracts were prepared, and 5- μ g samples were used in mobility shift assay with the palindromic NF- κ B site as a probe. (b) Serum-starved HeLa S3 were incubated for 60 min in the presence or absence of the protein kinase inhibitor staurosporin (150 nM) without any further treatment or in the presence of either nocodazole (NOC; 0.4 μ g/ml) or TPA (100 ng/ml). Nuclear extracts were prepared, and 5- μ g samples were used in a mobility shift assay with an NF- κ B-specific probe.

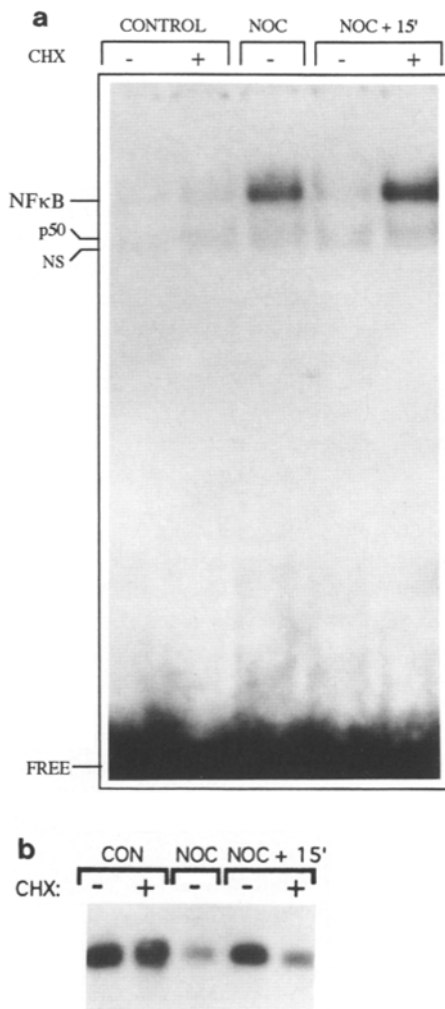


Figure 6. Microtubule repolymerization reverses NF- κ B activation by nocodazole. (a) Serum-starved HeLa S3 cells were incubated for 30 min in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX; 10 μ M) without any further treatment or in the presence of nocodazole (NOC; 0.4 μ g/ml) for 60 min. In two samples, cells were washed extensively with PBS and then replaced in medium containing or lacking cycloheximide for 15 min (NOC + 15'). Nuclear extracts were prepared, and 5- μ g samples were used in a mobility shift assay with an NF- κ B-specific probe. (b) 15- μ g cytoplasmic extracts derived from the same cells analyzed above were immunoblotted with a polyclonal antiserum to I κ B α . The regions of the autoradiogram corresponding to the specific I κ B α band is shown.

Exposure to Low Temperature Reveals Two Steps in NF- κ B Activation

To examine the effect of microtubule depolymerization on NF- κ B activity without using pharmacological agents, we incubated HeLa cells at 4°C for different time periods, and we examined the distribution of NF- κ B-binding activity between the cytosolic and nuclear fractions. Exposure to low temperature is known to cause depolymerization of microtubules (51). As shown in Fig. 7 a, incubation of HeLa cells at 4°C for ≥ 1 h elevated NF- κ B-binding activity in the cytoplasmic fraction but not in the nuclear fraction. Upon warming to 37°C, NF- κ B-binding activity appeared in the nuclear fraction within 30 min. By contrast, nocodazole treatment

led to rapid nuclear translocation of activated NF- κ B, even though a significant portion of activated NF- κ B remained in the cytoplasmic fraction. The cytoplasmic form of NF- κ B is unlikely to be derived from leakage from the nucleus during cell fractionation since the p50 homodimer was exclusively nuclear under all conditions. Nocodazole elicited a stronger activation of NF- κ B than cold depolymerization, and this difference is reflected in the extent of I κ B α degradation (Fig. 7 b; quantitation by phosphorimage analysis indicated a 12-fold decrease in NOC-treated cells compared to fourfold decrease in cold treated cells after 60 min of treatment). As expected, the effect of low temperature exposure on NF- κ B activity correlates with its effect on microtubule integrity. Immunoblotting of Triton-soluble and -insoluble fractions using anti- β -tubulin antibody revealed considerable depolymerization of microtubules within 30 min of incubation at 4°C and rapid recovery upon temperature upshift (data not shown). These results suggest that NF- κ B activation in response to microtubule depolymerization occurs in at least two steps. During the first step, NF- κ B activation occurs in the cytoplasm, and during the second step, it is translocated to the nucleus. Since low temperature is likely to inhibit nuclear transport, an energy- and temperature-dependent process (49, 54), it causes the accumulation of free NF- κ B in the cytoplasm. Activation in the cytoplasm is consistent with our previous results, where irradiation with ultraviolet C was used to activate NF- κ B in enucleated cells (21).

Microtubule Depolymerization Activates NF- κ B-dependent Gene Expression

To examine whether the induction of NF- κ B-DNA-binding activity in vitro correlates with increased NF- κ B-dependent transcription in vivo, HeLa cells were transfected with a CAT reporter gene controlled by a truncated *c-fos* promoter upstream to which two copies of the κ B-binding site were inserted, 2 κ B-CAT. As shown in Fig. 8, nocodazole treatment stimulated CAT activity fourfold, while treatment with TPA resulted in a 8.5-fold stimulation of CAT activity. Nocodazole or TPA treatment did not induce expression of a CAT reporter containing two copies of a mutated version of the κ B site upstream of the *c-fos* promoter, 2 κ m κ B-CAT. As was shown in the mobility shift assays (see Fig. 3), nocodazole had no effect on expression of an AP-1-dependent reporter, -73Col-CAT (2), whereas TPA stimulated expression of this reporter fivefold (Fig. 8). These results are consistent with the notion that microtubule depolymerization results in specific activation of NF- κ B-dependent gene expression.

Discussion

Over the years, many examples have been found that demonstrate a link between cell shape changes, the cytoskeleton, and alterations in the program of gene expression (6). However, the signal transduction pathways by which the cytoskeleton can influence gene expression have remained a mystery. The series of experiments described above demonstrate a pathway by which a major component of the cytoskeleton, the microtubule network, can affect the nuclear transcriptional machinery by modulating the activity of a specific transcription factor. We show that a variety of agents and treatments that depolymerize microtubules cause rapid and efficient activation of the transcription factor NF- κ B and

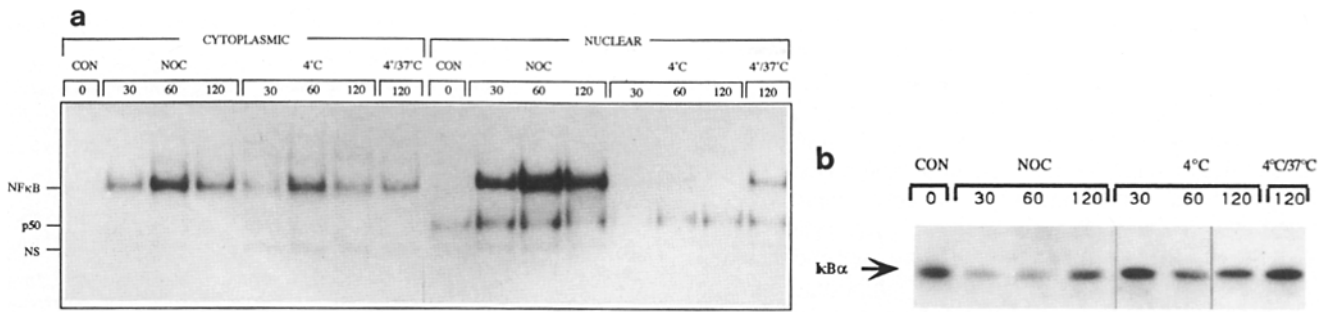


Figure 7. Activation of NF- κ B by cold shock. HeLa S3 cells were incubated in control medium at either 37°C (CON, 0 time point) or 4°C, or in the presence of 0.4 μ g/ml nocodazole (NOC) at 37°C for the indicated time periods (in minutes). An additional plate of cells was incubated at 4°C for 120 min and then shifted to 37°C for 30 min (4–37°C). Cells were harvested, and cytoplasmic and nuclear extracts were prepared. (a) The level of NF- κ B-binding activity was determined by mobility shift assays using nuclear extracts (5 μ g) and cytoplasmic extracts (derived from the same number of cell equivalents as 5 μ g of nuclear extract) and a radiolabeled palindromic NF- κ B probe. The migration positions of the protein–DNA complexes formed by NF- κ B, the p50 homodimer, and the nonspecific DNA binding protein (NS) are indicated. (b) The level of I κ B α protein in the cytoplasmic extracts (15 μ g/lane) was determined by immunoblot with an antiserum to I κ B α . Only the portion of the autoradiogram corresponding to the I κ B α band is shown.

thereby activate transcription of NF- κ B-responsive genes. Taxol, a microtubule-stabilizing agent, blocks the induction of NF- κ B by microtubule-disrupting agents and β -lumlcolchicine, an analogue of colchicine that does not disrupt microtubules, does not activate NF- κ B. These results strongly suggest that the observed induction is a direct and specific response to microtubule depolymerization. Treatment of cells with cytochalasin D, which causes depolymerization of actin filaments, did not result in effective NF- κ B activation. Therefore, NF- κ B activation is not caused by nonspecific and general disruption of the cytoskeleton. In addition, the DNA-binding activities of two other transcription factors, Spl and AP-1, were not affected by the state of microtubule polymerization.

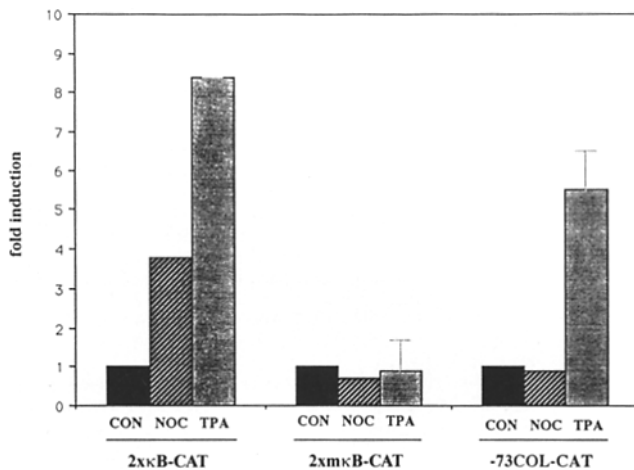


Figure 8. Nocodazole induces expression of an NF- κ B-dependent reporter gene. HeLa S3 cells were transfected with the indicated reporter constructs, as described in Materials and Methods. The fold induction was calculated relative to the level of expression of each reporter in untreated cells. The nocodazole (NOC; 0.4 μ g/ml) and TPA (100 ng/ml) treatments were for 12 h, and they were initiated 24 h after transfection. The results shown represent the averages of four separate experiments for the 2 κ κ B-CAT reporter and three experiments for the 2 κ m κ B-CAT and -73 Col-CAT reporters that were used as controls.

Microtubule-depolymerizing agents were previously shown to induce the expression of at least two genes, coding for uPA (11, 38) and IL-1 β (23, 44), both of which are also inducible by phorbol esters and inflammatory mediators, and are known to be regulated by NF- κ B (29, 33). The mechanism of uPA transcription of colchicine was shown to involve AP-1 (13), while its activation by phorbol ester involves NF- κ B (29). We were not able to detect a considerable increase in AP-1-binding activity after microtubule depolymerization (Fig. 3), but binding activity to an oligonucleotide corresponding to the NF- κ B-binding site in the uPA promoter (29) is induced to the same extent as the palindromic NF- κ B-binding activity after nocodazole treatment (Rosette, C., unpublished result). The mechanism of IL-1 β induction by microtubule depolymerization is unknown, although NF- κ B may certainly be involved. It was shown that colchicine induces the expression of IL-1 β mRNA and protein in human monocytes (23, 44), and we found that NF- κ B-binding activity to an oligonucleotide corresponding to the NF- κ B-binding site located in the IL-1 β promoter (33) is induced in monocytes treated with colchicine (Fig. 4 c). Interestingly, I κ B α was cloned as a rapidly induced transcript in human monocytes after adherence (30), a cellular state that is accompanied by changes in the organization of the cytoskeletal network (8).

In addition to phorbol esters, NF- κ B activity is induced by TNF- α , IL-1, bacterial lipopolysaccharide, and in a few cases, by serum growth factors (reviewed in references 3, 28, 39). It is of interest that all of these agents are known to induce reorganization of the cytoskeleton (1, 47). Thus, it is possible that partial or selective depolymerization of microtubules by any of these agents could be an intermediate in the signaling pathway leading to activation of NF- κ B, or act in synergy with other more conventional pathways, such as the activation of yet-to-be identified protein kinases, thought to activate NF- κ B through phosphorylation of I κ B (3, 39). So far, however, we found that pretreatment with taxol does not block the induction of NF- κ B by either TPA (Fig. 4 a), TNF- α , or serum (Rosette, C., unpublished results). Therefore, none of these signals appear to rely on microtubule reorganization.

How does depolymerization of microtubules lead to acti-

vation of NF- κ B? As with other inducers of NF- κ B such as TPA and TNF- α (4, 12, 32, 58), microtubule disruption by drugs and by cold treatment lead to I κ B degradation (Figs. 4 b and 7 b). The critical step leading to I κ B degradation may be its phosphorylation (Karin, M., and T. Hunter, manuscript submitted for publication). Two protein kinases, MAP kinase (57) and protein kinase A (44) were shown to be activated by colchicine. The kinase that phosphorylates I κ B in vivo has not been identified. Nevertheless, we find that incubation with the general protein kinase inhibitor, staurosporin, prevents the activation of NF- κ B by nocodazole. Hence, a protein kinase may be involved in this signaling event as well. Alternatively, degradation of I κ B may be triggered by other mechanisms, such as ubiquitination. Interestingly, ubiquitin and ubiquitin activating enzyme are localized to microtubules (48, 59). Whatever the mechanism of I κ B degradation, our observations that the level of NF- κ B-binding activity induced by microtubule-disrupting drugs and by cold treatment correlates well with the degree of I κ B α disappearance and that resuppression of NF- κ B-binding activity upon microtubule repolymerization is dependent on new protein synthesis, suggest it is likely regulated by changes in I κ B level.

Although the exact biochemical process by which depolymerization of microtubules leads to activation of NF- κ B remains to be elucidated, the present findings establish a role for NF- κ B in sensing changes in the state of the cytoskeleton and converting them to changes in gene activity. While the present work has relied on agents and treatments that cause general depolymerization of microtubules, it is likely that NF- κ B and related transcription factors may actually become activated in response to depolymerization of a small and specific subset of the entire microtubule network. Since such cytoskeletal changes are likely to be induced by cell-substrate and cell-cell interactions, this process would provide a signal transduction pathway by which these physical interactions can modulate gene expression and thereby affect the differentiated phenotype.

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