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Involvement of Nuclear Factor κB in c-Myc Induction by Tubulin Polymerization Inhibitors

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ABSTRACT

We showed previously that microtubule disassembly by vinblastine induces the proto-oncogene c-myc in epithelial mammary HBL100 cells (Bourgarel-Rey et al., 2000). In this study, we demonstrate that vinblastine treatment in these cells, in contrast to what was observed with the colon adenocarcinoma cell line HT29-D4, activated the transcription factor NFκB, which has been involved in c-myc regulation. The microtubule disassembly also induced IκB degradation. Using transient transfection analysis, we show that the *trans*-activation of c-myc by vinblastine was decreased when NFκB binding sites on c-myc promoter were mutated. Additionally, we demonstrate that microtubule dissolution *trans*-activated a thymidine kinase-CAT construct containing an NFκB binding site at −1180 to −1080 bp relative to the P1 promoter. Thus, vinblastine up-regulates the enhancer activity of the NFκB binding site. These results suggest that microtubule disassembly induced by vinblastine can *trans*-activate the c-myc oncogene through NFκB. Taking into consideration the paradoxical roles of both c-myc and NFκB in proliferation or apoptosis, this data reveals the complex action mechanism of this microtubule interfering agent.

The cytoskeleton is involved in many aspects of cellular function, such as cell movement, muscle contraction, phagocytosis, and mitosis. Its structural alterations affect cell physiology in many ways. Some studies suggest a link between cell shape changes, cytoskeleton dynamics, and alterations of gene expression. Through direct interaction with members of transduction pathways, the cytoskeleton may control the localization of signaling molecules and thus regulate gene expression (Ben-Ze'ev, 1991). In particular, cytoplasmic microtubules represent a major element of the cytoskeleton and this network may be involved in intracellular signaling. Because of their dynamic instability, microtubules are subject to constant remodeling (Jordan and Wilson, 1998). Agents that alter microtubule assembly, cell cycle progression during mitosis, and changes in the cytoskeleton (Jordan and Wilson, 1998) induce a range of cellular responses. For example, some of these agents stimulate mitogen-activated protein kinases (Schmid-Alliana et al., 1998; Wang et al., 1998; Guise et al., 2001) and modulate gene expression [cyclooxygenase-2 (Subbaramaiah et al., 2000), tumor necrosis factor-α, interleukin-1, CHUK, etc. (Moos and Fitzpatrick, 1998)]. We showed previously (Bourgarel-Rey et al., 2000) that drug-mediated inhibition of microtubule polymerization accompanied the up-regulation of the nuclear c-myc gene. The c-myc proto-oncogene is known to play a critical role in basal cell growth and deregulation of the expression of this gene is involved in the development of a variety of tumors (Bishop, 1991). The product of the c-myc gene is a nuclear phosphoprotein that has been implicated in the regulation of cell differentiation, apoptosis, and cell proliferation (Kato and Dang, 1992; Thompson, 1998). Transient induction of a low level of c-myc mRNA follows the growth activation of virtually all the quiescent untransformed cells examined (Kelly et al., 1983). This increase in expression is required for cells to enter S phase. The c-Myc protein contains multifunctional regions including an N-terminal *trans*-activation domain and a C-terminal domain that is required for heterodimerization. These data supported the model of c-Myc functioning as a transcription factor whose activity can be regulated by its protein binding partners. The transcription of c-myc is influenced by multiple *cis*-elements located on the enhancer, some of which have been shown to bind nuclear proteins (Hay et al., 1987). Among them, NFκB has two binding sites on the c-myc promoter (Ji et al., 1994). NFκB was originally identified as a mediator of activation of the κ-light-chain gene in B cells (Sen and Baltimore, 1986). Classical NFκB is a heterodimer consisting of two subunits, p50 and p65, each of which is also capable of homodimerizing and binding to specific targets on its own. In the resting state, NFκB is complexed in the cytoplasm with the inhibitory

V.B.-R. and S.B. contributed equally to this work

ABBREVIATIONS: TAX, paclitaxel; VLB, vinblastine; NCZ, nocodazole; FBS, fetal bovine serum; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assays; CAT, chloramphenicol acetyltransferase; bp, base pair(s); MTOC, microtubule organizing center.

protein IκB, which regulates its cellular localization, DNA binding, and transcription activities. IκB contains ankyrin repeats that are thought to form binding sites for both integral membrane proteins and tubulin (Rosette and Karin, 1995).

One plausible way in which the cytoskeleton can affect nuclear gene expression is by modulating the activity of transcription factors that reside in the cytoplasm of unstimulated cells as an inactive form and migrate to the nucleus in response to various stimuli. NFκB, which preexists as a latent complex in the cytoplasm of unstimulated cells (Baeuerle and Baltimore, 1988), constitutes such a factor.

In this work, we studied the involvement of NFκB in c-myc induction by antimicrotubule agents. Among the drugs that affect tubulin, paclitaxel (TAX) stabilizes the microtubules, whereas vinblastine (VLB) and nocodazole (NCZ) inhibit their polymerization. We studied the activation of NFκB when treating cells with antimicrotubule agents in HBL100 and HT29-D4 cell lines. These cell lines were selected because we described previously (Bourgarel-Rey et al., 2000) that microtubule disassembly induced c-myc expression in HBL100 but had no effect on c-myc expression in HT29-D4 cells. The NFκB activation was compared with this modulation of c-myc expression. Thus, we have tested whether c-myc gene up-regulation induced by tubulin polymerization inhibitors (vinblastine and nocodazole) resulted from NFκB activation. With the present data, we demonstrated that this c-myc induced stimulation is partly mediated by NFκB.

Materials and Methods

Cell Culture. Human colon adenocarcinoma HT29-D4 cells and normal human epithelial mammary HBL100 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and L-glutamine. Cells (80% of confluence) were starved 24 h with FBS-free medium and then treated with various drugs. This FBS starvation led to the greatest possible decrease in the basal c-myc expression. This culture condition was used for all experiments (NFκB activation, IκB degradation, myc promoter activation, and microtubule disassembly visualization).

Reagents. Stock solutions of paclitaxel (Sigma, St Quentin, France) and nocodazole (Sigma) were prepared at a 10 mM concentration in dimethyl sulfoxide. Vinblastine (1 mM) (Lilly, St Cloud, France) was prepared in sterile distilled water and kept frozen until use. Cycloheximide (Sigma) was solubilized at 10 mg/ml in water just before experiments and was used at 50 μg/ml.

Cytotoxicity Assay. Cytotoxicity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay as described previously (Carles et al., 1998).

Preparation of Nuclear Extracts. After 1.5 h treatment with the different drugs, cells (5×10^6 HT29-D4 cells or 5×10^6 HBL100 cells) were washed with cold Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin (BSA) and with cold PBS. They were resuspended in 0.4 ml of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and protease inhibitors) and kept in ice. Then, 25 μl of 10% Nonidet P-40 was added. Nuclei were separated from cytosol by centrifugation. Nuclei pellets were resuspended in 50 μl of buffer B (50 mM HEPES, 50 mM KCl, 0.3 M NaCl, 0.1 mM EDTA, 12% glycerol, and protease inhibitors). Lysates were separated by centrifugation. Protein concentration was measured with a commercial kit (Micro-BCA; Pierce, Rockford, IL).

Electrophoretic Mobility Shift Assays. EMSA were performed by incubating 4 μg of the nuclear extract in 20 μl of binding buffer B [0.25 μg of poly(dIdC) and 20 μg BSA]. Then, 40 fmol of ^{32}P-labeled oligonucleotide probe (50,000 cpm) was added.

The oligonucleotide probe used was the NFκB tandem binding site of HIV-1 enhancer: 5′-GAGAAGGGACTTTCCGCTGGGGACTTTCCAG-3′. For supershift assays, the probe free reaction mixture was incubated with anti-NFκB (p50 or p65) antibodies (Euromedex, Souffelweyersheim, France) and the ^{32}P-labeled oligonucleotide probe was then added. The mixture was separated by electrophoresis in a native 5% polyacrylamide gel, which was then dried and autoradiographed.

Western Blot Analysis. After stimulation, the culture medium was removed and cells were washed with cold PBS and lysed for 30 min at 4°C in radioimmunoprecipitation assay buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1% Triton, 1 mM EDTA, and protease inhibitors) without BSA. Cell lysates were centrifuged for 15 min (15,000*g* at 4°C). Supernatants were stored at −80°C. Samples (20 μg) were heated at 100°C for 5 min in Laemmli sample buffer containing reducing agent, separated by 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Nonspecific sites were blocked by 3-h incubation with PBS, 0.1% Tween 20, and 5% dried milk at room temperature. Membranes were then incubated overnight at 4°C with anti-IκBα antibody (Euromedex) (1:2000 dilution) in blocking solution, washed four times, and incubated with a horseradish peroxidase-labeled secondary antibody (1:2000 dilution) for 1 h at room temperature. The bound horseradish peroxidase was revealed with the luminescence ECL system.

Plasmid Constructs. pMpCAT (Avigan et al., 1990), which contains c-myc promoter sequences from nucleotides −2328 to −936, was obtained from D. Levens (National Institutes of Health, Bethesda, MD).

pMpCATΔ1Δ2 was derived from pMpCAT by mutation (underlined below) of the two binding sites of NFκB using the Quickchange site directed mutagenesis kit (Stratagene, Amsterdam, the Netherlands). The primers used were: NFκB upstream site (−1130 bp), US MMYC S 5′-CGGTTTTTTTCACAAG*CC*TCTCTGCTCACTCCCCC-3′ and US MMYC AS 5′-GGGGGAGTCAGCAGAGA*GG*CTTGTGAAAAAAACCG-3′; NFκB exon 1 site (+ 460 bp), DS MMYC S 5′-CTGCCCATTTGG*CC*ACACTTCCCCGCCGC-3′ and DS MMYC AS 5′-GCGGCGGGGAAGTGT*GG*CCAAATGGGCAG-3′. The mutations were confirmed by digestion using restriction enzymes. The Δ1 mutation led to the disappearance of one of the two *Bsa* I restriction sites present in the wild-type plasmid. The Δ2 mutation creates a second *Msc* I restriction site. The pMpCAT and pMpCATΔ1Δ2 plasmids were prepared and purified at the same time in exactly the same conditions. They were quantified by densitometry and agarose gel electrophoresis.

$pBLCAT_2$, which contains the chloramphenicol acetyl transferase (CAT) gene under the control of the thymidine kinase promoter, was obtained from M. Daujat (Daujat et al., 1996). Up-pBLCAT was obtained from $pBLCAT_2$ after addition of a sequence containing the upstream NFκB binding site on the c-myc promoter (between residues −1180 bp and −1080 bp relative to the P1 promoter). This allowed testing of the regulatory enhancer activity of this sequence.

The selected region was amplified by PCR using 50 ng of $pBLCAT_2$ DNA and Pfu polymerase. We introduced *Xba*I and *Hin*dIII restriction sites (underlined sequences, respectively) into the primers for cloning in $pBLCAT_2$ plasmid polylinker. The primers used were: Myc NF upstream sense, 5′-GAAAGGTCTAGAGCGTCCGGG-3′; and Myc NF upstream antisense, GGACTTCAAGCTTGGGGCAAGTGGAGAGCT-3′. The amplified product (123 bp) was further digested by *Xba*I and *Hin*dIII.

Activation of c-Myc Transcription: CAT Assays. Cells in six-well plates were transiently transfected in log phase using lipofectin (Life Technologies, Cergy Pontoise, France). They were incubated with 1 μg of plasmid DNA for 18 h. Cells were stimulated with drugs 24 h later and harvested after 48 h of treatment. The CAT expression was then evaluated by the amount of CAT protein using the CAT enzyme-linked immunosorbent assay system (Roche Molecular Di-

agnostics, Meylan, France). Statistical significance was evaluated with the use of analysis of variance. The transfection efficiency between dishes was verified by transfecting cells with pCMV/βgal. β-Galactosidase activity was evaluated by spectrofluorometry as described previously (Bourgarel-Rey et al., 2000).

Immunofluorescence Microscopy. Cells grown on glass coverslips were washed twice in PEM/PEG buffer containing 100 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, and 4% of PEG 8000. Soluble proteins were extracted for 90 s in PEM buffer containing 0.5% Triton X-100 and 1 mM GTP, then rinsed 3 times for 5 min with PEM/PEG/GTP. Cells were fixed in formaldehyde-dimethyl sulfoxide (3.7%/1%). They were then stained with anti-α-tubulin (1/400 dilution) and anti-mouse immunoglobulin fluorescein-linked whole antibody (1/20 dilution) (Amersham Pharmacia Biotech, Saclay, France) as described previously (Carles et al., 1999).

Results

Activation of NFκB by Anti-Microtubule Agents. The effect on NFκB activity of cell treatment with antimicrotubule agents was examined on nuclear extracts by EMSA using consensus NFκB binding sequences. We established in HBL100 cells a kinetic analysis of NFκB activation after a 10 μM VLB treatment (Fig. 1) and determined that the effect was maximal at 1.5 h. We then studied the effect of various doses of VLB. Although the effect of 10 nM VLB remained unclear, 100 nM VLB led to NFκB nuclear translocation. The HT29-D4 cells were found to be unresponsive to 10 μM the antimicrotubule agents TAX, VLB, and NCZ (Fig. 2A). However, treatment of HBL100 cells with 10 μM VLB or NCZ (another tubulin polymerization inhibitor) led to the activation and nuclear translocation of NFκB (Fig. 2B). Most of the activated NFκB complexes consisted of heterodimer p50/p65 because anti-p50 (Fig. 2C) and anti-p65 antibodies (data not shown) induced supershifts. No such NFκB activation was found with 10 μM TAX (Fig. 2B).

Rate of IκB Renewal Is Altered by Anti-Microtubule Agents. The degradation of the inhibitor protein IκB is a critical event in the process of NFκB activation. Most of the NFκB-inducing agents analyzed so far cause degradation of IκB within minutes (Beg et al., 1993; Henkel et al., 1993).

We evaluated, by Western blot analysis, the time course of IκBα expression after antimicrotubule agents treatment. Because IκBα resynthesis occurs quickly after degradation, this transient degradation can only be observed in the presence of a protein synthesis inhibitor (cycloheximide). Figure 3A shows that in HT29-D4, no change of IκBα immunostaining level was found with any of the drugs tested (TAX, VLB, and NCZ). In HBL100 cells (Fig. 3B), however, although no change was found with TAX, 10 μM VLB and NCZ induced an early decrease in IκBα. In HBL100 cells, we found that induction of NFκB binding activity by vinblastine or nocoda-

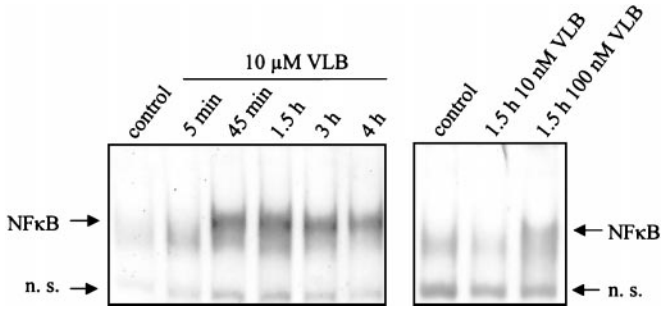

Fig. 1. Kinetic analysis and dose-effect of NFκB activation by VLB treatment in HBL100 cells. Nuclear extracts from HBL100 cells untreated (control), treated with 10 μM VLB for 5 min, 45 min, 1.5 h, 3 h, and 4 h or treated 1.5 h with 10 nM and 100 nM VLB. NFκB activation was determined by EMSA with a consensus NFκB binding site. The migration positions of NFκB protein-DNA complex (NFκB), and nonspecific protein-DNA complex (n.s) are indicated.

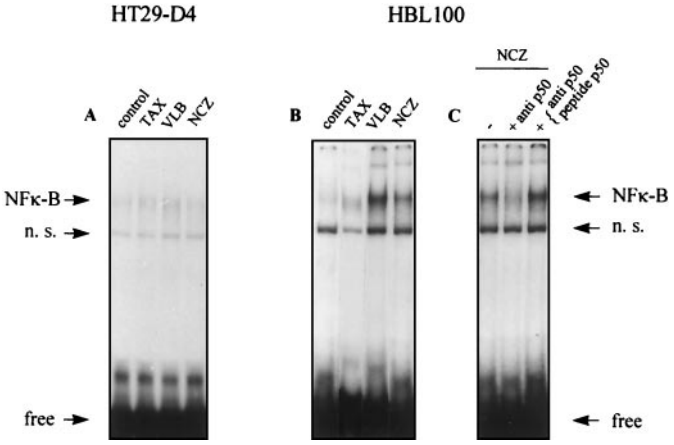

Fig. 2. Activation and nuclear translocation of NFκB according to the cell line and drugs used. Nuclear extracts from HT29-D4 (A) or HBL100 (B and C) cells untreated or treated for 1.5 h. Both cell lines were treated with 10 μM TAX, VLB, and NCZ. NFκB activation was determined by EMSA with a consensus NFκB binding site. The migration positions of NFκB protein-DNA complex (NFκB), a nonspecific protein-DNA complex (n.s) and the unbound probe (free) are indicated. For the identification of NFκB complexes (C), nuclear extracts were incubated with antibody specific for NFκB subunit p50 or with this antibody and exogenous peptide p50.

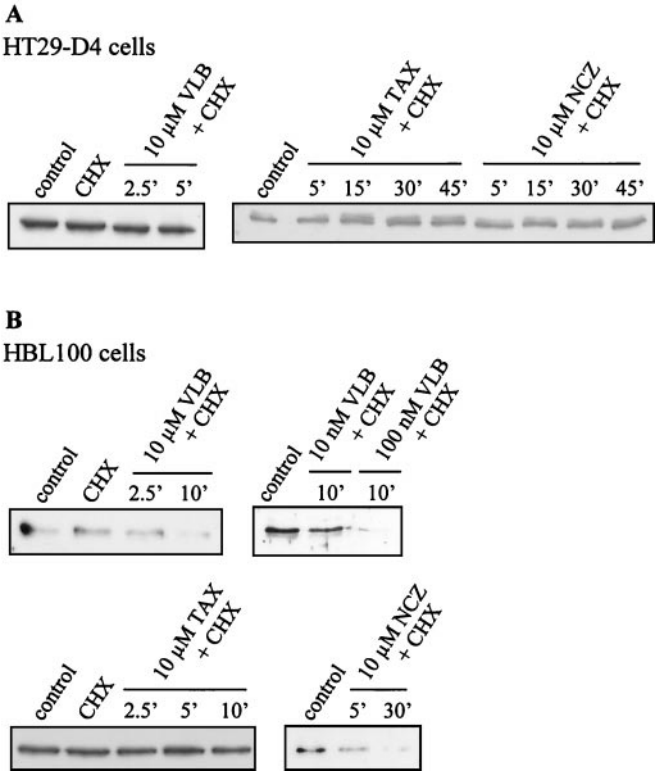

Fig. 3. Representative Western blot analysis of IκB expression. A, HT29-D4 cells were pretreated with cycloheximide (CHX) (50 μg/ml) for 1 h then treated with 10 μM VLB, TAX, and NCZ. B, HBL100 cells were pretreated with CHX (50 μg/ml) for 1 h then treated with 10 μM, 10 nM and 100 nM VLB, and with 10 μM TAX or NCZ for the times indicated.

zole correlates with the disappearance of IκBα. This observation was dose-dependent: no noticeable IκBα degradation with low VLB dose (10 nM) and important degradation with 100 nM.

Mutation of NFκB Binding Sites Affects the VLB-Induced *trans*-Activation of c-Myc Promoter. To assess whether the c-myc *trans*-activation by VLB that we described previously (Bourgarel-Rey et al., 2000) was caused by a VLB-induced change of NFκB binding to the c-myc promoter, site-directed mutations of c-myc promoter were inserted into each NFκB site. These mutations, which convert two guanines into two cytosines (Fig. 4A), were generated using the pMpCAT construct. Ji et al. (1994) showed by EMSA that NFκB binding to these mutated sequences was dramatically decreased.

Transient transfections of HBL100 cells with the pMpCAT mutated construct (pMpCATΔ1Δ2) resulted in decreased ability of VLB to induce CAT expression compared with that obtained with the wild-type c-myc promoter. As reported on Fig. 4B, the wild-type construct was induced 7.3-fold, whereas the mutated construct was induced only 4.2-fold. This significant ($p < 0.01$) decrease shows that the interaction of NFκB with c-myc promoter was involved in the *trans*-activation of the c-myc gene by VLB.

VLB Up-Regulates the Enhancer Activity of the NFκB Site. Thus, VLB was still able to activate, although to a lesser extent, the mutated c-myc promoter. One possibility is that VLB could activate other transcription factors involved in c-myc regulation. Indeed, multiple putative binding sites for transcription factors other than NFκB [E2F (Hiebert et al., 1989), Sp1 (Desjardins and Hay, 1993), and YY-1 (Riggs et al., 1993), etc.], are present in this region of the c-myc gene (between residues −2238 and +936, containing the upstream sequence, all of exon I, and a part of intron I). To avoid the interference of other transcription factors and to ascertain whether the observed induction of c-myc transcription is specifically related to the activation of NFκB, we investigated the effects of VLB on CAT expression using HBL100 cells transfected with the Up-pBLCAT$_2$, a CAT reporter gene construct containing the NFκB "upstream" site (100 bp corresponding to the NFκB binding site of c-myc promoter located between residues −1180 bp and −1080 bp relative to the P1 promoter). VLB induced a 3-fold increase of CAT expression, although no induction was observed after transfection of cells with pBLCAT$_2$ control construct.

Visualization of Microtubule Disassembly after VLB Treatment. We visualized the microtubules by immunofluorescence after VLB treatment to relate their polymerization state to IκB degradation and NFκB activation. Similar results were obtained with HBL100 (Fig. 5) or HT29-D4 cells (data not shown). The low VLB dose (10 nM), which did not affect microtubule polymerization after 5 min of treatment (Fig. 5B), led to only partial disassembly even after 5.5 h of incubation; the microtubules near the microtubule organizing center (MTOC) persisted and were still visible (Fig. 5D). When cells were treated with high VLB dose (10 μM), the microtubule disassembly was noticeable after 5 min (Fig. 5E). The disassembly of microtubules was obvious after 90 min (Fig. 5F) and complete after 5.5 h (Fig. 5G). At these times, paracristals were formed. We also studied an intermediate dose (100 nM), which led to results similar to those with 10 μM concerning microtubule disassembly, but did not induce paracristal formation (data not shown).

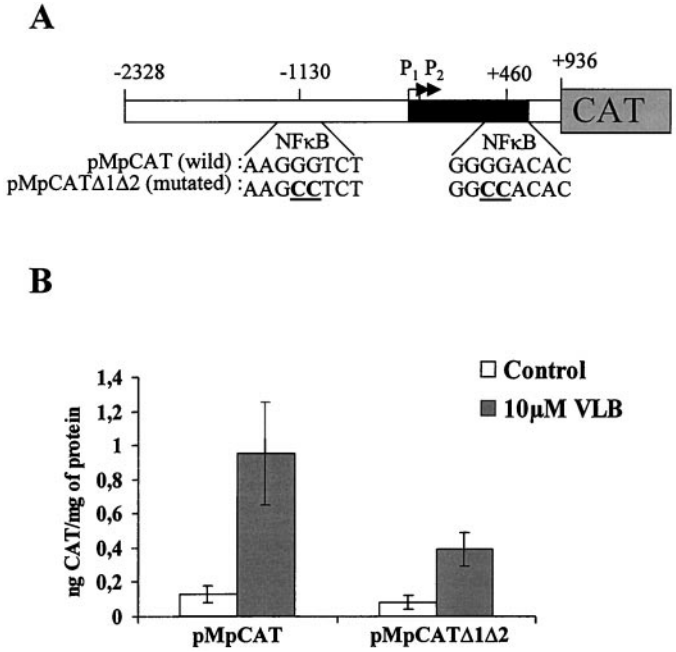

Fig. 4. Effect of mutated NFκB sites on c-myc promoter activation by vinblastine. A, diagram of c-myc promoter-CAT constructs used for transfection experiments. The location of the two NFκB binding sites is indicated. B, transient transfection analysis of c-myc promoter-CAT constructs (wild-type, pMpCAT; mutated type, pMpCAT Δ1Δ2). Evaluation of CAT expression by CAT enzyme-linked immunosorbent assay analysis in HBL100 transfected cells, untreated (control) and treated with 10 μM VLB. $n = 10$, $p < 0.01$.

Discussion

In this study, we provide evidence, first of all, for a correlation between c-myc expression and NFκB activation by antimicrotubule agents. We have shown previously that a high dose of tubulin polymerizing inhibitors, as opposed to a low dose, induced c-myc expression (Bourgarel-Rey et al., 2000). This induction was detectable at 100 nM but reached a maximum at 10 μM. Using a kinetic analysis, we determined a maximal induction between 4 and 6 h after 10 μM VLB addition (data not shown). Our present results suggested that NFκB activation could be related to c-myc expression because: 1) in HBL100, high doses of tubulin assembly inhibitors stimulated NFκB and c-myc expression whereas low doses had no effect and 2) microtubule-stabilizing agents in HBL100 cells, and all antimicrotubule agents in HT29-D4, did not stimulate NFκB or c-myc expression either.

We did not find any NFκB activation in HT29-D4 cells. This is in agreement with Jobin et al. (1997) who showed that NFκB nuclear translocation by interleukin-1 is less important and delayed in HT29 compared with Caco-2, another colon carcinoma cell line, suggesting a cell specificity. These different behaviors in response to antimicrotubule agents are not caused by different drug sensitivity. We evaluated the IC_{50} values of the different drugs in both cell lines and found similar results (10 nM for VLB and 25 nM for TAX).

These effects of tubulin assembly inhibitors on HBL100 cells could be related to those described by Rosette and Karin (1995). These authors showed, in HeLa S3 cells, that disassembly of microtubules activated NFκB and induced NFκB-

dependent gene expression. They suggested that the cytoskeleton could affect gene expression by modulating the activity of a specific transcription factor such as NFκB. NFκB is modulated by changes in the cytoskeleton state and converts them into changes in gene activity.

NFκB is a factor mediating regulation of c-myc gene and is activated by agents known to enhance the expression of c-myc. In particular it has been shown that NFκB is activated by interleukin-1, tumor necrosis factor-α, phorbol ester, and serum (Sen and Baltimore, 1986; Osborn et al., 1989; Baldwin et al., 1991). All of these agents can induce c-myc expression. More directly, Duyao et al. (1992) demonstrated the importance of the interaction of NFκB with c-myc promoter elements in the *trans*-activation process of the c-myc gene by HTLV-1 *tax*.

We demonstrated in the present study that the activation of c-myc transcription described previously (Bourgarel-Rey et al., 2000) is mediated by NFκB. Indeed, mutations within the NFκB binding sites of c-myc promoter decreased the ability of VLB to stimulate the transcriptional activity of the gene, suggesting a direct role of NFκB activation in the induction of c-myc transcription. The direct involvement of NFκB is demonstrated in the reporter gene assay, where treatment of cells with VLB stimulated activity from a transfected NFκB binding site-linked CAT plasmid.

Microtubule-perturbing agents were shown previously to induce the expression of two genes known to be regulated by NFκB (uPA and interleukin-1β) (Botteri et al., 1990; Ferrua et al., 1990). Dissolution of microtubules could be an intermediate of the signaling pathway leading to activation of NFκB. The NFκB activation requires degradation of its inhibitory protein IκB. Phosphorylation of IκB by specific activated protein kinase tags it for proteolytic degradation. This allows the nuclear translocation of activated NFκB, whereupon gene expression is activated. We have shown that tubulin polymerization inhibitors induced IκB degradation in HBL100 cells. Moreover, it has been shown that colchicine (another tubulin polymerization inhibitor) activated two protein kinases (mitogen activated protein kinase and protein kinase) (Shinohara-Gotoh et al., 1991; Manie et al., 1993), both of which could be implicated in IκB phosphorylation and then NFκB activation (Zhong et al., 1997; Norris and Baldwin, 1999).

Alternatively, degradation of IκB in response to microtubule disassembly may be triggered by a mechanism other than its phosphorylation. Indeed, Crepieux et al. (1997) demonstrated that the IκB protein interacted physically with DLc-1, a cytoskeleton-associated dynein light chain protein. Both DLc-1 and IκB interacted with the microtubular network, particularly with the MTOC (Crepieux et al., 1997). We visualized in HBL100, by immunofluorescence analysis, the disassembly of the microtubule network after VLB treatment. With high VLB doses, the disorganization of microtubules was complete and MTOC disappeared, which could explain the observed IκB degradation leading to NFκB activation and c-myc induction. We observed that this microtubule disassembly began early with high-dose VLB (10 μM), as opposed to a low dose, which had no effect on IκB, NFκB, or c-myc. It is important to correlate IκB degradation with microtubule disassembly because its degradation is the first step of our signaling pathway. This early microtubule disassembly was confirmed by Gajate et al. (2000), who observed that microtubule disassembly was noticeable with an incubation as short as 15 min and became practically complete after 1 h of incubation with colchicine. In the same way, Yujiri et al. (1999) showed that nocodazole disrupted microtubules after 30 min of incubation. This microtubule disassembly coincided with mitogen-activated protein kinase kinase kinase 1 activation (Yujiri et al., 1999) and was in agreement with data demonstrating that vincristine induced JNK (c-Jun N-terminal kinase) activation in MCF-7 cells for 15 min (Srivastava et al., 1999). Given this information, if IκB degradation is caused directly by microtubule disassembly, it must be assumed that a partial disassembly is sufficient. All these data suggest that VLB's perturbation of cellular microtubules leads to c-myc induction through NFκB activation. This activation requires IκB degradation, which could take place either after the well known phosphorylation at two specific residues, serine 32 and 36, or more directly in response to microtubule disassembly. Thus, microtubule or-

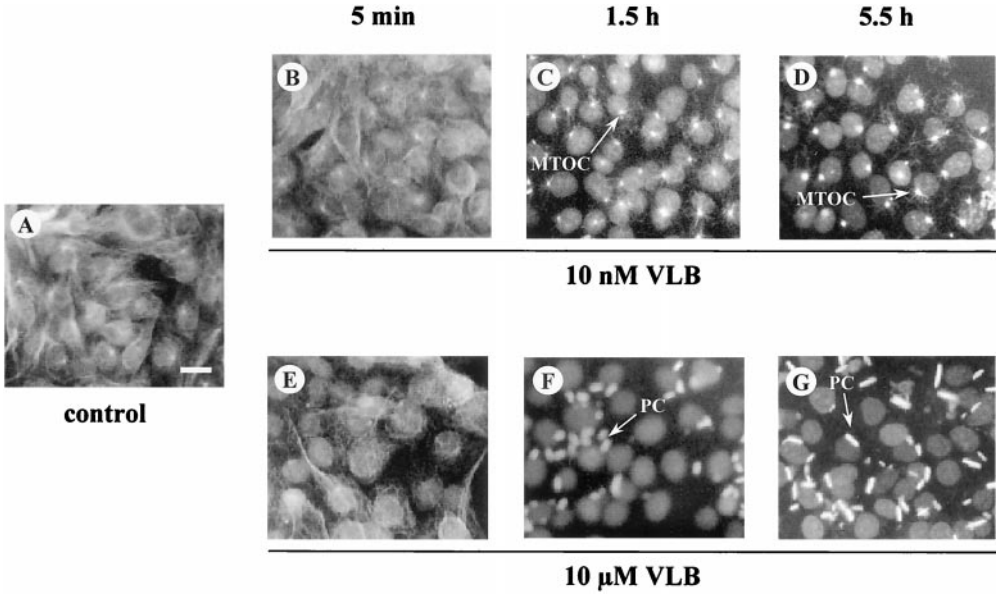

Fig. 5. Visualization of the effect of VLB on microtubule polymerization by indirect immunofluorescence staining. HBL100 untreated cells, control (A); HBL100 treated cells with 10 nM VLB for 5 min (B), 1.5 h (C), and 5.5 h (D); with 10 μM VLB for 5 min (E), 1.5 h (F), and 5.5 h (G). PC, paracristals. Scale bar, 20 μm.

ganization may be involved in the signal transduction leading to the activation of c-myc via NFκB. Activation of NFκB has been described as being involved in apoptosis, playing either an antiapoptotic or a proapoptotic role. It seems that the role of NFκB as a promoter or attenuator of cell death may ultimately depend on both the cell type and the nature of the apoptosis-inducing stimulus (Baichwal and Baeuerle, 1997). In the same way, it is now well known that the oncogene c-myc could, paradoxically, act on cell growth or death depending on the presence or absence of growth factors (Fuhrmann et al., 1999). Our data on c-myc activation by VLB mediated by NFκB concerns a new pharmacologic mechanism of this antimicrotubule agent. Indeed, vinblastine induces an early signal consisting in IκB degradation, NFκB activation, and then c-myc activation in HBL100 cells grown without growth factors. The involvement of c-myc and NFκB in apoptosis or proliferation are still debatable. The signaling pathway of such a treatment should be investigated further to resolve definitively the proliferation or apoptotic role of both myc and NFκB in this type of cell.

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