NF-κB Promotes Survival During Mitotic Cell Cycle Arrest

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Running title

NF-κB and cell survival.
Summary

By activating the mitotic checkpoint anti-microtubule drugs such as nocodazole cause mammalian cells to arrest in mitosis and then undergo apoptosis. Microtubule depolymerisation is rapid and results in the activation of the transcription factor NF-κB and induction of NF-κB-dependent gene expression. However, the functional consequence of NF-κB activation has remained unclear. Evidence has accumulated to suggest that NF-κB transcriptional activity is required to suppress apoptosis. In the present study we confirm and extend previous findings that microtubule depolymerisation leads to the rapid activation of NF-κB and test the hypothesis that the induction of NF-κB regulates cell survival during mitotic cell cycle arrest in order to define its role. Using a range of functional assays we have shown that microtubule depolymerisation correlates with the activation of IKKα and IKKβ, the phosphorylation, ubiquitination and degradation of IκBα, the translocation of native p65 (RelA) into the nucleus and increased NF-κB transcriptional activity. By inhibiting either the activation of the IKK’s or the degradation of IκBα, we find that the level of apoptosis is significantly increased in the mitotically-arrested cells. Inhibition of NF-κB signalling in the non-mitotic cells did not affect their survival. We establish that although NF-κB is activated rapidly in response to microtubule depolymerisation its cell survival function is not required until mitotic cell cycle arrest when the mitotic checkpoint is activated and apoptosis is triggered. We conclude that NF-κB may regulate the transcription of one or more anti-apoptotic proteins that may regulate cell survival during mitotic cell cycle arrest.
INTRODUCTION

By perturbing microtubule dynamics certain anticancer drugs cause mammalian cells to arrest in mitosis through activation of the spindle checkpoint (or mitotic checkpoint) (1). Generally, mitotic arrest is then followed by apoptosis (2,3). However, the biochemical mechanisms by which the anticancer drugs induce toxicity are not clearly understood. We have recently demonstrated that anticancer drugs such as nocodazole, vincristine and vinblastine (microtubule depolymerising agents) and taxol (a microtubule stabilising agent) activate intracellular signalling pathways that have opposing effects on cell survival (4). Another signal transduction pathway that is activated by these anticancer drugs involves the transcription factor nuclear factor (NF)-κB (5-7). However, the functional consequence of NF-κB activation by either microtubule depolymerising or stabilising drugs remains unknown.

NF-κB belongs to the Rel family of transcription factors that regulate genes involved in immune and inflammatory responses, cell cycle progression, apoptosis and oncogenesis (8,9). In mammals the Rel family comprises p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB and c-Rel. The NF-κB subunits homo- and heterodimerise to form transcription factor complexes with a range of DNA-binding and activation potentials. In unstimulated cells NF-κB exists in the cytoplasm as an inactive complex through association with one of several inhibitory molecules including IκBα, IκBβ, p105/IκBγ, IκBε and p100. NF-κB can be activated by a range of stimuli including inflammatory cytokines, phorbol esters, lipopolysaccharide, viruses, UV light and a variety of mitogens (10). These stimuli activate the IκB kinase (IKK) complex either directly or indirectly through activation of upstream protein kinases.
such as NF-κB-inducing kinase (NIK) (11,12), PKB (13,14), MEKK1, MEKK2 and MEKK3 (15-18). The IKK complex itself comprises two catalytic subunits, IKK-α, and IKK-β, and a structural component called IKKγ [or NEMO (NF-κB essential modulator) (19-22). The activated IKK phosphorylates the inhibitory κB proteins on either serine residues 32 and 36 of IκBα or serine residues 19 and 23 of IκBβ. This phosphorylation targets the IκB’s for ubiquitin-dependent degradation through the 26S proteasome complex, resulting in the release and nuclear translocation of NF-κB (23).

Recent studies provide compelling evidence that NF-κB suppresses cell death in response to a variety of apoptotic stimuli (9). For example, the disruption of genes encoding components of the NF-κB signalling pathway results in early embryonic lethality with the mouse embryos displaying increased apoptosis in various organs and tissues (9). Candidate anti-apoptotic genes targeted by NF-κB include the mitochondrial membrane stabilising proteins Blf-1 (24) and Bcl-xL (25,26), the caspase 8 inhibitor FLIP (27), the immediate early response gene IEX-1L (28) and the inhibitor of apoptosis proteins (IAP’s) cIAP1, cIAP2 and XIAP (29,30). Recent studies suggest that NF-κB can also inhibit TNFα-induced apoptosis by inhibiting activation of the c-Jun NH2-terminal kinase (JNK) via a poorly defined mechanism involving increased expression of either GADDβ or X-IAP (31,32). In this report we show that NF-κB is activated in HeLa cells in response to both microtubule depolymerising and microtubule stabilising anticancer agents. We also show that inhibition of NF-κB using three independent approaches selectively increases the susceptibility of mitotically-arrested cells to anticancer drug-induced apoptosis. Our
data suggest that NF-κB activation is linked to cell survival through the activation of NF-κB-responsive anti-apoptotic genes and may represent an important survival mechanism during mitotic cell cycle arrest.
MATERIALS AND METHODS

Cell culture and preparation of cell extracts
Human cervical carcinoma cells (HeLa) were cultured as described previously (33). To obtain mitotically-arrested cells, an asynchronous population of HeLa cells was treated with either nocodazole (3 µM), taxol (1 µM), vincristine (1 µM) or vinblastine (1 µM) for various times (0-30 h). Mitotic cells were collected by mechanical shake-off, washed in Dulbecco’s phosphate-buffered saline (DPBS) and lysed in the appropriate buffer depending on the protein kinase being assayed as described previously (33). Radio-immunoprecipitation (RIPA) buffer containing 1% v/v Triton X-100 was used to prepare cell lysates for immunoblotting with the IκBα antibodies. Cells remaining attached to the plates after shake-off were washed x3 with DPBS (containing either nocodazole [3 µM], taxol [1 µM], vincristine [1 µM] or vinblastine [1 µM]) and lysed by scraping into the appropriate buffer. Cell lysates were centrifuged at 14,000 g for 10 min at 4°C and the protein content of the supernatant determined using the Coomassie Protein Assay Reagent (Perbio Science UK Ltd, Cheshire, UK) before normalisation and solubilisation in x2 SDS-PAGE sample buffer.

Antibodies
IKKα, IKKβ, IκBα, anti-haemagglutinin epitope tag (HA) and phosphospecific histone H3 that was used as a marker for mitotic cells (Santa Cruz, California, USA). Anti-FLAG (M2), Cy3-conjugated anti-FLAG (M2), anti-γ-tubulin, anti-β-actin, HRP-conjugated goat anti-mouse, HRP-conjugated goat anti-rabbit and HRP-conjugated mouse anti-goat (Sigma, Dorset, UK). Anti-M30 which detects caspase cleavage cytokeratin 18 (34) (Roche Diagnostics Ltd, East Sussex, UK). Alexa Fluor 488 goat
anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 rabbit anti-
mouse IgG (Molecular Probes, Oregon, USA). Phosphospecific anti-IκBα (New
England Biolabs UK Ltd, Hertfordshire, UK). Mouse anti-Pk-Tag (Serotec, Oxford,
UK).

**Plasmids**
The plasmid expressing haemagglutinin (HA)-tagged ubiquitin was provided by Dirk
Bohmann (Scripps, CA, USA). Plasmids expressing both wild type Pk-Tag IκBα and
mutant (S32A/S36A) Pk-Tag IκBα were provide by Ron Hay (University of St.
Andrews, Scotland, UK). Mammalian constructs expressing the wild type IKK’s
(Flag-tagged), the Flag-tagged mutant IKK’s (K44A) and the construct for expressing
recombinant glutathione-S-transferase (GST)-IκBα fusion protein (amino acids 0-
200) were provided by David Goeddel (Tularik, CA, USA [19]).

**Immunoprecipitation and Western blot analysis**
Immunoprecipitations were performed as described previously (35). The
immunoprecipitated proteins and the cell extracts were resolved by SDS-PAGE and
electroblotted onto Hybond-C nitrocellulose membrane using a Hoeffer semi-dry
blotting apparatus (Amersham Biosciences, Piscataway, NJ). Immunoreactive
proteins were visualised using ECL (Amersham Biosciences).

**Protein kinase assays**
Immunecomplex kinase assays for the IKK’s were performed as described
previously (35) using GST-IκBα as substrate.
**Transfection of HeLa cells**

HeLa cells were plated at a density of approximately $5 \times 10^5$ cells per well in a 6 well plate or at $1 \times 10^5$ cells per well for coverslips in an 8 well plate. 18-24h later the cells were transfected with 1-4 µg of DNA using FuGENE 6 according to the manufacturers instructions (Roche Diagnostics Ltd). At 18-24h after transfection the cells on the coverslips were fixed in cold (-20°C) methanol, washed X2 with DPBS and then processed for immunofluorescence microscopy as described below. The cells in the 6 well plates were either washed x2 with DPBS and lysed in the appropriate buffer to make cell extracts or collected (floating and attached cells) for the determination of apoptosis as described above.

**Reporter assays**

For transient transfection of reporter plasmids, cells were plated in 12-well plates and the following day cells were co-transfected with a luciferase reporter plasmid containing either 3xNF-κB binding sites (Stratagene, CA, USA) in combination with the internal control CMV Renilla luciferase plasmid (provided by Anne Willis, University of Leicester). Luciferase activities of reporter plasmids were measured using the Dual-Luciferase Reporter Assay System (Promega UK, Southampton, UK).

**Immunofluorescence microscopy**

As described previously (4).

**Peptide synthesis and purification**

The amino acid sequence of both the wild type and the mutant (W739A, W741A) NEMO-binding domain (NBD) peptide fused to the Antennapedia homeodomain was
as described (36). Crude synthetic NBD peptides (wild type and mutant) were synthesised by Pepceuticals Ltd., (Leicester, UK). They were further purified using reverse-phase HPLC chromatography (Agilent Technologies UK Ltd., Stockport, Cheshire, UK) and concentrated by freeze-drying. The quality of the purified peptides was assessed using an analytical HPLC column (Perkin Elmer Instruments, Buckinghamshire, UK) and by electrospray mass spectrometry on a Micromass Platform-II instrument (Waters Ltd., Manchester, UK).

**Densitometry**

 Autoradiograms were scanned and quantified using a β-Imaging Computing Densitometer (Molecular Dynamics, Little Chalfont, UK) with MD ImageQuant software (version 3.3).

**Reagents**

 Nocodazole, Taxol, Vincristine, Vinblastine, MG-132 (N-CBZ-leu-leu-leucinal) and ALLN (N-acetyl-leu-leu-norleucinal) were all obtained from Sigma. All other reagents were of analytical grade and obtained from Sigma.
RESULTS

NF-κB activation in response to anti-microtubule drugs is mediated by the IKK and IκB signalling pathway.

To investigate whether microtubule depolymerization activates NF-κB signalling we treated exponentially growing HeLa cells with nocodazole, a reversible microtubule depolymerising compound (1). Cytoplasmic extracts were assayed for the activation of both IKKα and IKKβ using immunecomplex kinase assays. As shown in Fig.1A, nocodazole treatment rapidly activated (within 15 min) both IKKα (1.8-fold activation above the basal level) and IKKβ (2.1-fold above the basal level). Both IKKα and IKKβ were increasingly activated for upto 6h after nocodazole addition. From 12h onwards we were able to separate the nocodazole-treated cells into a mitotic population (as assessed by increased phosphorylation of histone H3; [37]) and an attached, non-mitotic population (as assessed by low phosphorylation of histone H3). As shown in Fig.1A, both IKKα and IKKβ were highly activated in the mitotically-arrested cells (approximately 10- to 14-fold above basal levels respectively) in comparison to the attached cell population (2.5- to 2.1-fold above basal levels respectively). The activation of either IKKα or IKKβ could not be attributed to changes in their expression levels as indicated by immunoblots of the cytoplasmic extracts with the IKK-specific antibodies (Fig.1A). The activation of both IKKα and IKKβ by nocodazole correlated with microtubule depolymerisation as determined by immunocytochemistry with an α-tubulin antibody (Fig.1B). Although considerable microtubule depolymerisation was observed within 15 minutes of nocodazole addition, a more homogeneous distribution of α-tubulin was observed between 1-6h
after nocodazole addition (Fig.1B). To determine whether the activation of both IKKα and IKKβ was nocodazole-specific we additionally treated exponentially growing HeLa cells with either vincristine or vinblastine (to depolymerise microtubules) or with taxol (to stabilise microtubules). All four treatments caused activation of both IKKα and IKKβ in the attached cells and to a greater extent in the mitotic population (Fig.1C) indicating that both microtubule depolymerisation and microtubule stabilisation can cause activation of the IKK’s.

The activation of both IKKα and IKKβ by nocodazole also correlated with the phosphorylation and degradation of IκBα. As shown in Fig.2A nocodazole treatment increased the phosphorylation of IκBα on serine 32. The increase in the phosphorylation of IκBα above basal levels was observed between 15 to 30 minutes after nocodazole treatment. The phosphorylation of IκBα was sustained for up to 6h and correlated with the degradation of IκBα. Our results also show that although the IKK’s are highly active in the mitotically-blocked cells (Fig.1A), the phosphorylation of IκBα was reduced in this cell population (Fig.2A). As observed with nocodazole, treatment of HeLa cells with either vincristine, vinblastine or taxol also resulted in the phosphorylation and degradation of IκBα (Fig.2B) although IκBα appeared to be degraded slightly less efficiently in the presence of taxol.

To determine whether the degradation of IκBα by nocodazole resulted in the nuclear translocation of NF-κB we analysed the intracellular distribution of native p65 (RelA) by immunocytochemistry. As shown in Fig.3A p65 was distributed uniformly throughout the cell prior to the addition of nocodazole but was found primarily in the
nucleus following nocodazole treatment. We performed a time-course analysis of the nuclear accumulation of p65 in the non-mitotic, attached cells following nocodazole treatment. Our results indicate that p65 translocated to the nucleus between 30 to 60 minutes after nocodazole treatment with maximum nuclear localization occurring 6 h after nocodazole treatment (Fig. 3B). Between 6 and 24 h after nocodazole treatment the number of cells showing a nuclear localization of p65 declined although this phenotype remained above basal levels. Control cells treated with DMSO alone did not show any change in the distribution of the p65 protein during the time-course examined. In mitotic cells (collected at 12-24 h after nocodazole treatment) p65 was distributed uniformly throughout the cytoplasm (data not shown).

To examine whether the nuclear accumulation of p65 (RelA) resulted in an increase in NF-κB-dependent transcription, HeLa cells were transfected with a luciferase reporter gene that is regulated by three tandem repeats of an NF-κB enhancer element (3 x pNF-κB-Luc). As shown in Fig. 4 nocodazole treatment stimulated a time-dependent increase in luciferase activity in both the attached and the mitotically-arrested cells. Taken together, our results suggest that the perturbation of microtubule dynamics leads to the activation of NF-κB-dependent gene expression through an IKK/ IκBα-dependent signalling pathway.

Dominant negative IKKα, IKKβ and non-degradable IκBα suppress nocodazole-induced activation of NF-κB and reduce the survival of nocodazole-blocked mitotic cells.
To determine whether NF-κB was involved in regulating the survival of nocodazole-arrested mitotic cells signalling defective mutants of both IKKα (K44A) and IKKβ (K44A) were used. Transfection of HeLa cells with MEKK1, a known activator of NF-κB (16) resulted in a 5-fold stimulation of luciferase activity in an NF-κB reporter assay (Fig.5A). The MEKK1 stimulated luciferase activity was comparable to that observed with either wild type (wt) IKKα (5.1-fold in the attached cells; 4.3-fold in the mitotic cells) or wt IKKβ (5.4-fold in the attached cells; 6.3-fold in the mitotic cells) after nocodazole treatment. However, in the presence of either IKKα (K44A) or IKKβ (K44A) both basal and nocodazole-stimulated luciferase activity was reduced when compared to their respective wild type controls [IKKα (K44A) % reduction: asynchronous cells 90%, attached cells 76% and mitotic cells 64%; IKKβ (K44A) % reduction: asynchronous cells 90%, attached cells 95% and mitotic cells 90%]. The reduction of luciferase activity by either dominant negative IKKα or IKKβ could not be attributed to variations in the expression levels of the recombinant proteins as shown by immunoblots of cell extracts with IKK-specific antibodies (Fig.5B). To determine the function of NF-κB signalling following nocodazole treatment, HeLa cells were transfected with either wild type IKKα or IKKβ or with either dominant negative IKKα or IKKβ. As shown in Fig.5C treatment of control, mock-transfected cells with nocodazole caused 27% of the mitotically-arrested cells to undergo apoptosis whereas the attached cells remained viable (2.8% apoptotic cells). Similarly, in cell expressing either wild type IKKα or IKKβ nocodazole treatment caused approximately 41% and 39.5% of the mitotically-arrested cells to undergo apoptosis respectively. However, in cells expressing either dominant negative IKKα or IKKβ nocodazole treatment resulted in 71% and 74% of the mitotically-arrested cells
undergoing apoptosis. Neither the wild type nor the dominant negative IKK’s greatly affected the survival of the nocodazole-treated attached cells.

To further investigate the relationship between NF-κB activation and the survival of nocodazole-arrested mitotic cells we transfected cells with either wild type IκBα or with a mutant, non-degradable IκBα (S32A/S36A, [38]). As shown in Fig.6A non-degradable IκBα reduced nocodazole-stimulated luciferase activity by 82% in the attached cells and by 78% in the mitotically-arrested cells when compared to wild type IκBα. The reduction in luciferase activity by the non-degradable IκBα could not be attributed to variations in the level of expression of the mutant IκBα when compared to the wild type protein (Fig.6B). Expression of non-degradable IκBα selectively increased the level of apoptosis in the nocodazole-arrested mitotic cells (79% apoptosis compared to 41% apoptosis in cells expressing wild type IκBα), without affecting the survival of the nocodazole-treated attached cells (Fig.6C).

**The NBD peptide inhibits nocodazole-induced activation of NF-κB and reduces the survival of nocodazole-blocked mitotic cells.**

As a second approach to suppress nocodazole-induced activation of NF-κB we used the NBD peptide, a known inhibitor of the IKK’s (36). Treatment of exponentially growing HeLa cells with nocodazole for 3h caused p65 to translocate to the nucleus in approximately 32% of the cells (Fig.7A). However, treatment of cells with 100µM NBD peptide reduced nocodazole-induced p65 translocation into the nucleus by 80% to near basal levels, whereas treatment of cells with a mutant NBD peptide caused a small, but statistically significant (p<0.05) increase in nocodazole-induced p65
translocation into the nucleus (Fig. 7A). The inhibition of p65 nuclear translocation by the NBD peptide was not restricted to nocodazole treatment but was also observed with other NF-κB-inducers such as TNF-α (data not shown). To determine the effect of the NBD peptide on the survival of the nocodazole-treated cells, exponentially growing HeLa cells were treated with nocodazole in the presence of varying concentrations of either the wild type or the mutant NBD peptide. As shown in Fig. 7B the wild type NBD peptide, but not the mutant peptide, caused a dose-dependent increase in apoptosis in the mitotically-arrested cells but not in the attached cell population.

**Proteasome inhibitors block nocodazole-induced activation of NF-κB and reduce the survival of nocodazole-blocked mitotic cells.**

Finally, we have used inhibitors of the 26S proteasome to inhibit nocodazole-induced degradation of IκBα and suppress activation of NF-κB (38-41). As shown in Fig. 8A treatment of exponentially growing HeLa cells with nocodazole resulted in the time-dependent degradation of IκBα. The nocodazole-induced degradation of IκBα was inhibited by the proteasome inhibitors MG132 or ALLN (42) but not the protease inhibitors leupeptin or PMSF (Fig. 8A). The inhibition of IκBα degradation by MG132 either in the absence (Fig. 8B, lane 4) or presence of nocodazole (Fig. 8B, Lane 3) correlated with the appearance of higher molecular weight forms of IκBα when compared to control, untreated cells (Fig. 8B, lane 1) or following treatment of cells with nocodazole only (Fig. 8B, lane 2). The low molecular weight bands (indicated by an asterisk) observed in the IκBα immunoblot have also been observed in a previous study (38) and are thought to represent intermediates in the degradation of IκBα.
These intermediate products of IκBα degradation were lost following nocodazole treatment (Fig.8B, lane 2) but their levels were maintained in cells treated with MG132 either in the presence or absence of nocodazole (Fig.8B, lanes 3 and 4). To further demonstrate that MG132 inhibits degradation of ubiquitinated IκBα, we co-transfected HeLa cells with HA-ubiquitin and PkTag-IκBα. Immunoblots of the anti-HA immunoprecipitates from control, untreated cells (Fig.8C, lane 1) indicated the presence of monoubiquitinated IκBα (approximately 48kD) when compared to native IκBα (approximately 40kD) (Fig.8C, lane marked ‘cell lysate’). Anti-HA immunoprecipitates from cells transfected with PkTag-IκBα alone indicated the presence of IgG bands only (Fig.8C, lane 5). Treatment with nocodazole resulted in the degradation of ubiquitinated IκBα (Fig.8C, lane 2) that was suppressed by MG132 (Fig.8C, lanes 3 and 4).

To determine the effect of MG132 on nocodazole-induced apoptosis HeLa cells were treated with either nocodazole or with both nocodazole and MG132. As shown in Fig.8D MG132 enhanced nocodazole-induced apoptosis as assessed by cleavage of cytokeratin 18. Quantitation of the data shown in Fig.8D indicated that MG132 caused a dose-dependent increase in nocodazole-induced cell death selectively in the mitotically-arrested cells and not in the attached, non-mitotic cells (Fig.8E). Similarly, ALLN but not PMSF or leupeptin, also enhanced nocodazole-induced apoptosis preferentially in the mitotic cell population. Treatment of cells with the inhibitors alone did not increase apoptosis significantly above basal levels. Together these results suggest that activation of NF-κB by microtubule depolymerising agents and the corresponding increase in NF-κB-dependent gene expression may be a mechanism by which NF-κB contributes to the survival of mitotically-arrested cells.
DISCUSSION

Previous work has clearly demonstrated that microtubule depolymerisation leads to the activation of NF-κB and NF-κB-dependent gene expression (5). What has remained unclear is the functional consequence of activating this particular signalling pathway. In this study we have identified a number of components in the NF-κB signalling pathway that are activated by anti-microtubule drugs and demonstrate that one functional consequence of activating this signalling pathway is to selectively aid the survival of mitotically-arrested cells.

We have shown in this study that a range of compounds that either depolymerise or stabilise microtubules can activate the NF-κB signalling pathway. Consistent with previous work (5) we have shown that the phosphorylation, ubiquitination and degradation of IκBα by the anti-microtubule drugs accompany the activation of NF-κB. Furthermore, we now demonstrate that the activation of NF-κB by the anti-microtubule drugs is also dependent on the activation of the IKK’s. Recent studies have shown that while both IKKα and IKKβ are critical for NF-κB-mediated gene expression, only IKKβ appears to be critical for IκB degradation (43-45). IKKα appears to regulate NF-κB-mediated gene expression independently of IκB through direct interaction with the promoters of NF-κB-responsive genes and phosphorylation of histone H3 (46,47). Our analysis of IKK activation following nocodazole treatment indicates that both IKKα and IKKβ are activated to an equal extent and that this activation is biphasic. The early activation of both IKK’s is readily reconciled with microtubule depolymerisation and their role in NF-κB-mediated gene expression through either an IκB-dependent or -independent pathway. The function of the late
activation of the IKK’s that we observe specifically in the mitotically-arrested cells is more difficult to reconcile with changes in gene expression as both transcription and translation are generally suppressed during mitosis (48,49). The identity of the protein kinase(s) that activate the IKK’s in mitotically-arrested cells and their potential function remains to be identified although there are data to suggest that the activation of the IKK’s in mitotically-arrested cells may be mediated by caspases. In mammalian cells the three death domain-containing proteins caspase-8, caspase-10 and MRIT (Mach-related inducer of toxicity) have been shown to activate IKK by a mechanism that involves an interaction between the prodomain of caspases and the IKK’s (50). This caspase-dependent mechanism of IKK activation would be consistent with the observation that caspase 8 is known to be active in taxol-arrested mitotic cells (51,52).

Although microtubule depolymerisation and not microtubule stabilization was originally reported to be the stimulus for NF-κB activation (5), subsequent studies have shown that the microtubule stabilising drug taxol can also activate NF-κB in mammalian cells (6,7,53,54). In the present study we have also shown that taxol can activate NF-κB as assessed by the increase in the phosphorylation and degradation of IκBα. Therefore, we suggest that it is not microtubule depolymerisation or stabilisation per se that may lead to NF-κB activation but rather a perturbation of microtubule dynamics. Another explanation for the activation of NF-κB by both microtubule depolymerising and microtubule stabilising drugs is that these compounds are thought to activate a common enzyme, such as PKC (6) that subsequently activates NF-κB. The mechanism by which PKC stimulates NF-κB is unclear but may involve the activation of the IKK’s (55).
The reported interaction between IκBα and a microtubule-associated dynein light chain protein (56) may provide the link between changes in microtubule dynamics and activation of NF-κB. It can be envisaged that the pool of microtubule-bound IκBα may act as a sensor of microtubule dynamics that is phosphorylated and degraded upon microtubule depolymerisation/stabilization. It is less clear how anti-microtubule drugs stimulate the activation of the IKK’s. NF-κB-inducers such as TNF-α, IL-1 or Fas activate the IKK’s through upstream protein kinases such as NIK (12) and the MAP3K kinases including MEKK1, 2 and 3 (15-18). There are data to indicate that MEKK1 is activated in response to microtubule depolymerisation (57-58). However, using immunocomplex kinase assays we have not observed the activation of MEKK1, MEKK2 or MEKK3 in HeLa cells upon nocodazole treatment (data not shown). Whether it is NIK or other protein kinases that activate the IKK’s in response to the anti-microtubule drugs remains to be determined.

While the precise biochemical mechanism by which perturbation of microtubule dynamics leads to the activation of NF-κB remains unclear the current study has established a role for NF-κB in regulating cell survival during mitotic cell cycle arrest. As outlined in the Introduction the prevalent hypothesis is that NF-κB functions as an anti-apoptotic factor. In some instances, however, NF-κB is reported to have a pro-apoptotic function (53,54, 59-62) suggesting that the precise effect of NF-κB may be cell and stimulus-specific. Our data are consistent with the majority view and assign a survival function for NF-κB. Anti-microtubule drugs cause cell to arrest in mitosis through activation of the mitotic checkpoint and as demonstrated in this study and in earlier work (1-4) the mitotically-arrested cells then undergo apoptosis. Cells that
have been treated with the anti-microtubule drugs for an equivalent time but that are not in M-phase do not undergo apoptosis, suggesting that activation of the mitotic checkpoint initiates the apoptotic response. The mechanism by which activation of the mitotic checkpoint then leads to cell death is not yet understood. We have recently demonstrated that both pro-apoptotic signals, such as p38 MAP kinase, and anti-apoptotic signals such as p21-activated kinase (PAK) are preferentially activated in the mitotically-arrested cells (4). Other work has shown that the anti-apoptotic protein bcl-2 becomes hyperphosphorylated during mitotic cell cycle arrest and may constitute a pro-apoptotic signal (63). In contrast, the rapid activation of NF-κB in response to microtubule perturbation may represent a survival signal that is activated prior to activation of the mitotic checkpoint. Whether the activation of NF-κB and the resulting transcription of anti-apoptotic genes are fortuitous, or a programmed response to microtubule perturbation that pre-empts the requirement for survival factors during subsequent mitotic arrest is presently unclear. As outlined in the Introduction there are a number of anti-apoptotic genes that are targeted by NF-κB. The identity of the NF-κB-regulated anti-apoptotic gene product(s) that contribute to cell survival in response to anti-microtubule drugs is currently under investigation.
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FIGURE LEGENDS

Figure 1.
Anti-microtubule drug-induced activation of the IKK’s correlates with microtubule depolymerisation. Exponentially growing HeLa cells were treated with either DMSO (t=0h represent asynchronous cells) or with nocodazole (3μM) for the indicated times. 12h after nocodazole treatment the cells were separated into a mitotic (Mit) and an attached population (Att). Specific antibodies were used to immunoprecipitate (IP) IKKα and IKKβ from the cell lysates for either immunocomplex kinase assays or Western blots with anti-IKKα, anti-IKKβ or anti-phospho histone H3 (a marker of mitotic cells). A sample of the 12h mitotic cell lysate was also incubated with protein-A sepharose beads alone (C= control IP). The autoradiograms of the protein kinase assays were scanned by densitometry and the fold activation of the IKK’s (compared to the asynchronous cells) is indicated. (B) An asynchronous population of HeLa cells was treated with nocodazole for the indicated times, fixed and stained with an α-tubulin antibody as described in Materials and Methods. (C). Exponentially growing HeLa cells were treated with either nocodazole (3μM), vincristine, vinblastine or taxol (all 1μM) for 12h and the cells separated into mitotic and attached populations prior to performing immunocomplex kinase assays and Western blots. Asynchronous cells (Asy). The fold activation of IKKα and IKKβ is indicated.

Figure 2.
Anti-microtubule drugs induce the phosphorylation and degradation of IκBα. (A) Exponentially growing HeLa cells were treated with nocodazole (3μM) and cell extracts prepared at the indicated times after nocodazole addition. After 12h the
cells were separated into mitotic and attached populations as described in Materials and Method. The cell extracts were immunoblotted with a phospho-specific IκBα antibody, an IκBα antibody, a γ-tubulin antibody or a phospho-specific histone H3 antibody. (B) Exponentially growing HeLa cells were treated with either nocodazole (3μM), vincristine, vinblastine or taxol (all 1μM) for 12h and the cells separated into mitotic and attached populations prior to immunoblotting with a phospho-specific IκBα antibody, an IκBα antibody, a γ-tubulin antibody or a phospho-specific histone H3 antibody.

Figure 3.
Nocodazole induces the translocation of p65 (RelA) into the nucleus. (A) Exponentially growing HeLa cells were either treated with nocodazole (3μM) for 6h or DMSO treated. The attached, non-mitotic cells were then fixed and immunostained with a specific antibody to detect the localization of native p65 (RelA) as described in Materials and Methods. Indirect immunofluorescence of p65 (left panels), DNA staining (middle panels) and the merged images (right panels). (B) Quantitation of the data shown in (A) at the indicated times following nocodazole treatment in the attached cell population. Control cells (Con) were treated with an equivalent volume of DMSO. At least 100 cells were counted in randomly selected fields for each time point and the bars represent the mean±SD of three independent experiments.

Figure 4.
Nocodazole induces expression of an NF-κB-dependent reporter gene. Exponentially growing HeLa cells were co-transfected with an NF-κB/luciferase
reporter construct (1µg) and a *Renilla* luciferase reporter construct (1µg) in duplicate. 24h after transfection the cells were treated with nocodazole (3µM) for the indicated times. Cell extracts were prepared after separation of the attached and the mitotic populations at the indicated times and the reporter activity measured as described in Materials and Methods. The fold increase in NF-κB luciferase activity was calculated relative to the expression of *Renilla* luciferase in DMSO-treated control cells at each time point. Each bar represents the mean±SD of three independent experiments.

Figure 5.
Dominant negative IKK’s inhibit the nocodazole-induced activation of NF-κB and potentiate nocodazole-induced apoptosis in mitotically-arrested cells. (A) Exponentially growing HeLa cells were transfected with either the wild type (wt) or dominant negative (K44A) IKKα or IKKβ, or wt MEKK1 (all 1µg) together with an NF-κB/luciferase reporter construct (1µg) and a *Renilla* luciferase reporter construct (1µg) in duplicate. 24h after transfection the cells were treated with either nocodazole (3µM) or with an equivalent volume of DMSO (Asy) for 12h. Cell extracts were prepared after separation of the attached (Att) and the mitotic (Mit) populations and the reporter activity measured as described in Materials and Methods. Each bar represents the mean±SD of three independent experiments. (B) Cell extracts prepared in (A) were immunoblotted with with either a FLAG antibody to assess expression of the IKK’s or with a γ-tubulin antibody. Mock transfected cells (C). (C) HeLa cells were transfected with either the wt or K44A IKKα or IKKβ. 24h after transfection the cells were treated with nocodazole (3µM) for 12h. The mitotic cells were removed by ‘shake-off’ and re-attached to poly-D-lysine-coated coverslips. The
mitotic and attached cells were fixed and immunostained with a Cy3-conjugated anti-FLAG antibody to identify the transfected cells and with the M30 antibody to identify the apoptotic cells as described in Materials and Methods. At least 100 FLAG-positive cells were counted in randomly selected fields and the bars represent the mean±SD of three independent experiments. Mock transfected cells (Control).

Figure 6.
Dominant negative IκBα inhibits the nocodazole-induced activation of NF-κB and potentiates nocodazole-induced apoptosis in mitotically-arrested cells. (A) Exponentially growing HeLa cells were transfected with either the wild type (wt) or non-degradable (S32A/S36A) IκBα (both 1µg) together with an NF-κB/luciferase reporter construct (1µg) and a Renilla luciferase reporter construct (1µg) in duplicate. 24h after transfection the cells were treated with either nocodazole (3µM) or with an equivalent volume of DMSO (Asy) for 12h. Cell extracts were prepared after separation of the attached (Att) and the mitotic (Mit) populations and the reporter activity measured as described in Materials and Methods. Each bar represents the mean±SD of three independent experiments. (B) Cell extracts prepared in (A) were immunoblotted with with either a PkTag antibody to assess expression of IκBα or with a γ-tubulin antibody. (C) HeLa cells were transfected with either the wt or S32A/S36A IκBα. 24h after transfection the cells were treated with nocodazole (3µM) for 12h. The mitotic cells were removed by ‘shake-off’ and re-attached to poly-D-lysine-coated coverslips. The mitotic and attached cells were fixed and immunostained with the PkTag antibody to identify the transfected cells and with the M30 antibody to identify the apoptotic cells as described in Materials and Methods. At least 100 FLAG-positive cells were counted in randomly selected fields and the
bars represent the mean±SD of three independent experiments. Mock transfected cells (Control).

Figure 7.
The NBD peptide inhibits nocodazole-induced translocation of p65 (RelA) into the nucleus and potentiates nocodazole-induced apoptosis in the mitotically-arrested cells. (A) Exponentially growing HeLa cells were pre-treated with 100µM of either the wild type (wt) or mutant NBD peptide or with an equivalent volume of DMSO (Control) for 2h prior to nocodazole treatment (3µM) for 6h. The cells were then fixed and immunostained with a specific antibody to detect the localization of native p65 (RelA) as described in Materials and Methods. At least 100 cells were counted in randomly selected fields for each time point and the bars represent the mean±SD of three independent experiments. *p<0.05 (ANOVA) compared to control nocodazole-treated cells. (B) Graph showing the effect of the NBD peptide on nocodazole-induced apoptosis in the mitotically-arrested cells. Exponentially growing HeLa cells were pre-treated with the indicated concentrations of either the wild type (wt) or mutant NBD peptide or with an equivalent volume of DMSO (Control) for 2h prior to nocodazole treatment (3µM) for 12h. The mitotic cells were removed by ‘shake-off’ and re-attached to poly-D-lysine-coated coverslips. The mitotic and attached cells were fixed and immunostained with the M30 antibody to identify the apoptotic cells as described in Materials and Methods. At least 100 cells were counted in randomly selected fields and the bars represent the mean±SD of three independent experiments. The percentage of apoptotic cells (mean±SD, n=3) following treatment with the NBD peptide alone for 12h was: DMSO control 2.4±0.8, 200µM NBD (wt) 4.4±2.3, 200µM NBD (mutant), 4.1±2.1.
Figure 8.

Proteasome inhibitors suppress the nocodazole-induced degradation of IκBα and potentiate nocodazole-induced apoptosis in the mitotically-arrested cells. (A) HeLa cells were pretreated with DMSO, 100µM ALLN, 5µM MG132, 100µM PMSF or 250µM leupeptin for 1h prior to nocodazole treatment (3µM) for the indicated times. The cell lysates were then immunoblotted with an IκBα antibody. (B) HeLa cells were pretreated with 5µM MG132 or an equivalent volume of DMSO for 1h prior to nocodazole treatment (3µM) for 6h. The cell lysates were then immunoblotted with either an IκBα antibody or a γ-tubulin antibody. The positions of the molecular weight markers (kD) are indicated on the left. The slower migrating and faster migrating forms of IκBα are indicated by the brackets and asterisk respectively. (C) HeLa cells were co-transfected with PkTag- IκBα (1µg) and HA-Ub (1µg) (lanes1-4) or with PkTag- IκBα alone (lane 5). 24h after transfection the cells were pretreated with 5µM MG132 or an equivalent volume of DMSO for 1h prior to nocodazole treatment (3µM) for 6h. The cell lysates were immunoprecipitated with an anti-HA antibody and the immunoprecipitated proteins immunoblotted with the PkTag antibody. The positions of the molecular weight markers (kD) are indicated on the left. The positions of the native and ubiquitinated IκBα and the cross-reacting IgG bands are indicated on the right. Cell lysate:cell lysate prepared from mock transfected cells. (D) HeLa cells were pretreated with 5µM MG132 or an equivalent volume of DMSO for 1h prior to nocodazole treatment (3µM) for 12h. The mitotic cells were recovered by ‘shake-off’ and reattached onto poly-D-Lysine-coated coverslips. The mitotic were fixed and immunostained with the M30 antibody to identify the apoptotic cells as described in
Materials and Methods. Indirect immunofluorescence showing M30 staining (upper panels), DNA staining (middle panels) and the merged images (lower panels). (E) A graph showing the effect of proteasome inhibitors on the survival of nocodazole-arrested mitotic cells. HeLa cells were pretreated with either DMSO or with the indicated concentrations of ALLN, MG132, PMSF or leupeptin for 1h prior to nocodazole treatment (3μM) for 12h. The mitotic cells were removed by ‘shake-off’ and re-attached to poly-D-lysine-coated coverslips. The mitotic and attached cells were fixed and immunostained with the M30 antibody to identify the apoptotic cells as described in Materials and Methods. At least 100 cells were counted in randomly selected fields and the bars represent the mean±SD of three independent experiments. In cells treated with the inhibitors alone for 12h the percentage of apoptotic cells was as follows (mean±SD, n=3): DMSO Control 1.9±1.1, 10μM MG132 3.8±1.5, 200μM ALLN 4.6±0.9, 200μM PMSF 3.0±0.5 and 500μM Leupeptin 3.3±0.4.
Fig. 1A

| Nocodazole treatment (h) | 0 | 0.25 | 0.5 | 1 | 3 | 6 | 12 | 18 | 24 | 30 | 12 | 18 | 24 | C |
|--------------------------|---|------|-----|---|---|---|----|----|----|----|----|----|----|---|
| IKKα IP                  |   |      |     |   |   |   | 12 | 18 | 24 | 30 |    |    |    |   |
| IKKα WB                  |   |      |     |   |   |   | 12 | 18 | 24 |    |    |    |    |   |
| Fold Activation          | 1 | 1.8  | 2.4 | 2.1 | 2.5 | 3.3 | 9.7 | 9.2 | 10.1 | 8.7 | 2.5 | 1.5 | 1.3 | 0 |
| IKKβ IP                  |   |      |     |   |   |   | 12 | 18 | 24 | 30 |    |    |    |   |
| IKKβ WB                  |   |      |     |   |   |   | 12 | 18 | 24 |    |    |    |    |   |
| Fold Activation          | 1 | 2.1  | 3.4 | 4.1 | 3.3 | 4.0 | 13.3 | 12.3 | 13.8 | 7.9 | 1.9 | 2.1 | 2.1 | 0 |
| Phospho-Histone H3 WB    |   |      |     |   |   |   |     |     |     |     |     |     |     |   |

Fig. 1B

Time (min) post Nocodazole addition

Fig. 1C

| 12h drug treatment | Asy | Noc | Tax | Vinb | Vinc | Mit | Att |
|--------------------|-----|-----|-----|------|------|-----|-----|
| IKKα IP            |     |     |     |      |      |     |     |
| IKKα WB            |     |     |     |      |      |     |     |
| Fold Activation    | 1   | 5.4 | 3.8 | 5.2  | 2.7  | 5.3 | 3.3 |
| IKKβ IP            |     |     |     |      |      |     |     |
| IKKβ WB            |     |     |     |      |      |     |     |
| Fold Activation    | 1   | 4.9 | 3.4 | 4.7  | 2.9  | 4.3 | 2.8 |
| Phospho-Histone H3 WB |     |     |     |      |      |     |     |

Mistry et al. 2003 Figure 1
Fig. 2A

| Nocodazole treatment (h) | TOTAL | MITOTIC | ATTACHED |
|-------------------------|--------|---------|----------|
| 0                       | 0.25   | 0.5     | 1        |
| 3                       | 6      | 12      | 18       |
| 6                       | 12     | 18      | 24       |
| 12                      | 12     | 18      | 24       |
| 18                      | 12     | 18      | 24       |
| 24                      | 12     | 18      | 24       |
| 30                      | 12     | 18      | 24       |

Phospho-IκBα (S32) WB
Total IκBα WB
γ-Tubulin WB
Phospho-Histone H3 WB

Fig. 2B

| 12h drug treatment | Noc | Tax | Vinc | Vinb |
|--------------------|-----|-----|------|------|
|                    | Asy | Mit | Att  | Mit  | Att  | Mit  | Att  |
| Phospho-IκBα (S32) WB |
| IκBα WB            |
| γ-Tubulin WB       |
| Phospho-Histone H3 WB |

Mistry et al. 2003 Figure 2
Mistry et al. 2003 Figure 3
Mistry et al. 2003 Figure 4
Fig. 5A

Luciferase activity (fold change)

| MEKK1 | IKKα (wt) | IKKα (K44A) | IKKβ (wt) | IKKβ (K44A) |
|-------|-----------|-------------|-----------|-------------|
| Asy   | Att       | Mit         | Asy       | Att         | Mit         |

Fig. 5B

**IKKα (wt)**

| C     | Asy | Att | Mit |
|-------|-----|-----|-----|

**IKKα (K44A)**

| C     | Asy | Att | Mit |
|-------|-----|-----|-----|

**Anti-Flag WB**

**Anti-γ-tubulin WB**

Fig. 5C

Apoptosis (%)

| Attached | Mitotic |
|----------|---------|
| Control  | IKKα (wt) | IKKα (K44A) | IKKβ (wt) | IKKβ (K44A) |

Nocodazole treatment (12h)

Mistry et al. 2003 Figure 5
Fig. 6A

Luciferase activity (fold change)

|       | Asy | Att | Mt  |
|-------|-----|-----|-----|
| IκBα (wt) |     |     |     |
| IκBα (S32A/S36A) |     |     |     |

Fig. 6B

Anti-PKTag WB

Anti-γ-tubulin WB

Fig. 6C

Apoptosis (%)

|                   | Attached | Mitotic |
|-------------------|----------|---------|
| Control           |          |         |
| IκBα (wt)         |          |         |
| IκBα (S32A/S36A)  |          |         |

12h Nocodazole treatment

Mistry et al. 2003 Figure 6
Fig. 7A

Nuclear translocation of p65 (%)

DMSO
Noc

(wt) (mutant)

Fig. 7B

Apoptosis (%)

NBD (µM) + Nocodazole

NBD(wt) NBD (mutant)

0 25 50 100 200
Fig. 8A: 

Nocodazole treatment (h)

| Treatment                  | 0  | 0.5 | 1   | 3   | 6   |
|---------------------------|----|-----|-----|-----|-----|
| Noc (3μM)                 |    |     |     |     |     |
| Noc + 5μM MG132           |    |     |     |     |     |
| 5μM MG132                 |    |     |     |     |     |
| Noc + 100μM ALLN          |    |     |     |     |     |
| 100μM ALLN                |    |     |     |     |     |
| Noc + 100μM PMSF          |    |     |     |     |     |
| 100μM PMSF                |    |     |     |     |     |
| Noc + 100μM Leupeptin     |    |     |     |     |     |
| 100μM Leupeptin           |    |     |     |     |     |

Fig. 8B: 

Anti-IκBα WB

Nocodazole -  +  +  -
MG132        -  -  +  +

Ub-IκBα

IκBα

Anti-γ-Tubulin WB

Fig. 8C: 

Anti-HA IP

| Treatment                  | 1  | 2  | 3  | 4  | 5  |
|---------------------------|----|----|----|----|----|
| PK-Tag IκBα               | +  | +  | +  | +  | +  |
| HA-Ub                     | +  | +  | +  | +  | -  |
| Nocodazole                | -  | +  | +  | -  | +  |
| MG 132                    | -  | -  | +  | +  | -  |

IgG

Ub-IκBα

Native IκBα

Cell Lysate

Mistry et al. 2003 Figure 8a-c
NF-κB promotes survival during mitotic cell cycle arrest
Pratibha Mistry, Karl Deacon, Sharad Mistry, Jonathan Blank and Rajnikant Patel

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