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PHORBOL ESTER-MEDIATED NF-KAPPA-B TRANSACTIVATION IS SELECTIVELY INHIBITED BY TAXOL

by

William John Spencer

This thesis was submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Department of Microbiology and Immunology

McGill University, Montreal

March 1998.

To Anouk Adam,

Thank you for your perseverance and strength.

To Arthur Guinness and John Walker,

Time takes out the fire, but leaves in the warmth.

Abstract

Recently, NF-κB activation has been shown to be directly influenced by the cytoskeletal environment. In an attempt to better understand and characterize cytoskeletal regulation of NF-κB a series of experiments were designed to determine whether the microtubule (MT) stabilizing agent taxol could affect NF-κB activation in the presence of different NF-κB inducers. Pretreatment of murine NIH 3T3 and human 293 cells with 5 μM taxol resulted in complete abolition of phorbol, 12-myristate, 13-acetate (PMA) mediated NF-κB activation including loss of DNA binding potential and reduced CAT reporter activity. Phosphorylation and turnover of IκBα was effectively abrogated in taxol pretreated COS-7 cells. However, taxol was not capable of preventing TNF-α induced NF-κB activation nor could taxol suppress IκBα inducible phosphorylation in TNF-α treated cells, suggesting TNF-α may function through a microtubule-independent pathway. In vitro kinase assays of PMA stimulated cells established that taxol could reduce activation of protein kinase C by 30%, establishing loss of PKC activity as a possible regulatory step in taxol-mediated suppression of NF-κB transactivation. Indirect immunofluorescence analysis revealed that PMA treated COS-7 cells underwent dramatic changes in cell morphology as well as depolymerization of MTs. These observations were similar to that seen for nocodazole treated cells, a known MT depolymerizing agent. In contrast, taxol blocked both nocodazole induced effects as well as PMA induced morphological changes. These findings establish a potential mechanism for taxol-mediated stability of MTs and inhibition of NF-κB activity, suggesting a link between the state of microtubule integrity and gene regulation.

Résumé

Il a récemment été montré que la régulation du cytosquelette joue un rôle direct dans l'activation de NF-κB. Afin de mieux connaître et comprendre la régulation de NF-κB par le cytosquelette, nous avons entrepris une série d'expériences dans le but de déterminer si le taxol, agent stabilisant des microtubules (MT), est capable d'affecter l'activation de NF-κB en présence de différents inducteurs. Dans des cellules NIH 3T3 prétraitées par 5 μM de taxol, l'activation de NF-κB induite par le phorbol, 12-myristate, 13 acétate (PMA) est complètement inhibée au niveau de la fixation à l'ADN et l'activité d'un gène indicateur CAT est réduite. De façon analogue, la phosphorylation ainsi que la néosynthèse et dégradation de IκBα sont inhibées dans les cellules COS-7 prétraitées par le taxol. Cependant, le taxol n'affecte ni l'activation de NF-κB ni la phosphorylation de IκBα induites par le TNF-α. Ce résultat suggère que le TNF-α n'agirait pas au niveau des microtubules. Grâce à des expériences de phosphorylation in vitro, nous avons montré que le taxol inhibe l'activation de la protéine kinase C de 30% après stimulation des cellules par le PMA, ce qui suggére que la PKC serait impliquée dans l'inhibition de NF-κB induite par le taxol. Nous avons observé par immunofluorescence indirecte que le traitement par le PMA de cellules COS-7 induit un important changement de morphologie ainsi que la depolymérisation des microtubules. Des résutats semblables ont été observés après traitement des cellules par le nocodazole, un agent connu pour son action de dépolymérisation des microtubules. Cependant, le taxol est capable de supprimer les effets induits par le nocodazole ainsi que les changements morphologiques provoqués par le PMA. Ces résultats mettent en évidence un mécanisme potentiel, par lequel le taxol agirait sur la stabilité des microtubule et l'inhibition de l'activité de NF-κB, suggérant l'existence d'un lien entre l'intégrité des microtubules et la régulation des gènes.

Acknowledgments

I would like to personally thank everyone in the Hiscott Lab past and present who helped me directly or indirectly with this project, your time and patience were well honored. These people include Carmela DeLuca, Hannah Nguyen, Louisa Petropolous, Christophe Heylbroeck, Nadine Pelletier, Yaël Mamane, Dr. Pierre Génin, Lindsay Teskey, Dr. Pascale Crépieux, Dr. Pierre Beauparlant, Normand Pepin, Sonia Cisternas, and Dana Zmeureanu.

I would personally like to recognize Hakju Kwon, my friend and confidante. Hakju's presence in the lab was a great relief as well as his prevailing support for my somewhat outlandish ideas. His outstanding abilities in and out of the lab are an admirable quality and I wish him well.

I would also like to thank Andy Wong, Nicolas Solban, Sylvie Wise, Nancy Tam, Tetsu Ishii, Dr. Fortunado Manganaro, André Paquin, and Juliana Gabor, my closest friends during the good, the bad, and the not so attractive... Cheers!.

Personal thanks go to Dr. Michele Algarté for translation of the abstract and to Dr. Rongtuan Lin for exceptional advice and technical assistance during my time here.

To my parents, thank you for taking care of my stipend, without your love, support, and patience this work would never have been accomplished.

Finally, I would like to thank my supervisor John Hiscott. Dr. Hiscott's lab gave me one of the best opportunities to learn firsthand the finer details and demands of scientific research. Dr. Hiscott's critical review of all manuscripts and oral presentations were

invaluable. The caliber of excellence demanded by this lab has set the ground work for my future research.

Preface

The candidate was responsible for all the work presented in this thesis with exception of SDS gel electrophoresis separation of phosphorylated IκBα (Figure 17) which was performed by Hakju Kwon. As well, Figures 5, 6, 7A and 7B in the introduction were performed by Dr. Pascale Crépieux.

Part of the data presented in the thesis was submitted as the following manuscript pending publication:

Spencer, W., H. Kwon, P. Crepiéux, R. Lin, and J. Hiscott. (1998) Phorbol Ester Induced NF-kappa-B Activation is Selectively Inhibited by Taxol: A Role for PKC and the Microtubule Cytoskeleton.

Part of the data presented in the introduction was published as the following manuscript:

Crepiéux P., H. Kwon, N. LeClerc, **W. Spencer**, S. Richard, R. Lin, and J. Hiscott. (1997) IκBα physically interacts with a cytoskeleton-associated protein through its signal response domain. *Molecular and Cellular Biology.* **17:** 7375-7385.

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INTRODUCTION

1 The Cytoskeleton and Signal Transduction.

In recent years, a growing body of evidence has reinforced the idea that the cytoskeleton has the potential to regulate gene activation through a complex regulatory process involving structural cues in the cytoskeletal matrix and the effective balance of numerous populations of cytoskeletal-associated proteins (51,72,108,140,179). The transmission of information from the plasma membrane to the nucleus is associated with a number of rapid changes in cell physiology. In regards to cytoskeletal regulation of signaling pathways, the actin cytoskeleton has been the most extensively characterized (7,112,192).

Possibly the best characterized cytoskeletal system is that involving the Rho family of GTPases, an important class of actin regulatory proteins (192). This family of GTPases are important modulators of signal transduction pathways from extracellular stimuli to the cell nucleus and include at least three major pathways: JNK/SAPK, p38, and NF-κB (135,166). Three major members of this family, namely RhoA, CDC42, and Rac-1 are responsible for a number of biological activities such as actin organization (96), gene expression (79,185), and cellular transformation (13,86,134). Rho is believed to participate in signaling responses that lead to the formation of actin stress fibers and focal adhesions (143,144). Actin stress fibers are associated with integrins through an interface with focal adhesion complexes at the inner surface of the plasma membrane. In this manner, Rho is capable of participating in the regulation of cell morphology (131), cell aggregation (175), cell motility (169), and smooth muscle contraction (70). As well, rho proteins are also involved in cellular transformation in a similar fashion to that of the Ras proto-oncogene (136,137). Furthermore, Rac1 and RhoA are important elements in Ras-mediated transformation although their role is not as clearly defined.

Rho proteins also regulate the mitogen-activated protein kinases (MAPK) (96,109,201). This second class of regulators is also important in actin cytoskeleton regulation during proliferation and differentiation (140). In human B lymphocytes, the extracellular regulated kinases (ERK/MAP) are activated by protein kinase C (PKC) (163) upon cross-linking of the B-cell antigen receptor (25). Activated MAPK subsequently phosphorylates $p90^{rsk}$ an important downstream link between cytoplasmic and nuclear signaling (197). The regulation and transmission of MAPK activated signals is initiated by assembly of actin in the vicinity of the cytoplasmic domain of the B-cell antigen receptor (112), a process believed to be mediated by tyrosine kinase activation (111). A unique characteristic of MAPK is that it is associated with the microtubule cytoskeleton (141) and recent evidence now suggests the microtubule cytoskeleton is a key regulator of actin polymerization. Fluid shear stress-mediated signal transduction, a process by which endothelial cells are capable of transducing mechanical force into biological responses brings together both actin and microtubule regulation with MAPK as a central player (78). During fluid shear stress, an integrin-mediated response, causes stress fiber and focal adhesion formation, mediated by a PKC-activated MAPK pathway that also requires modulation of the microtubule cytoskeleton (203). In light of the above evidence it is becoming clear that the Rho family of GTPases, actin regulation, MAPK, and the microtubule cytoskeleton represent a complex signaling pathway not previously defined.

2. The Microtubule Cytoskeleton.

2.1 Dynamic Aspects of Microtubule Biology.

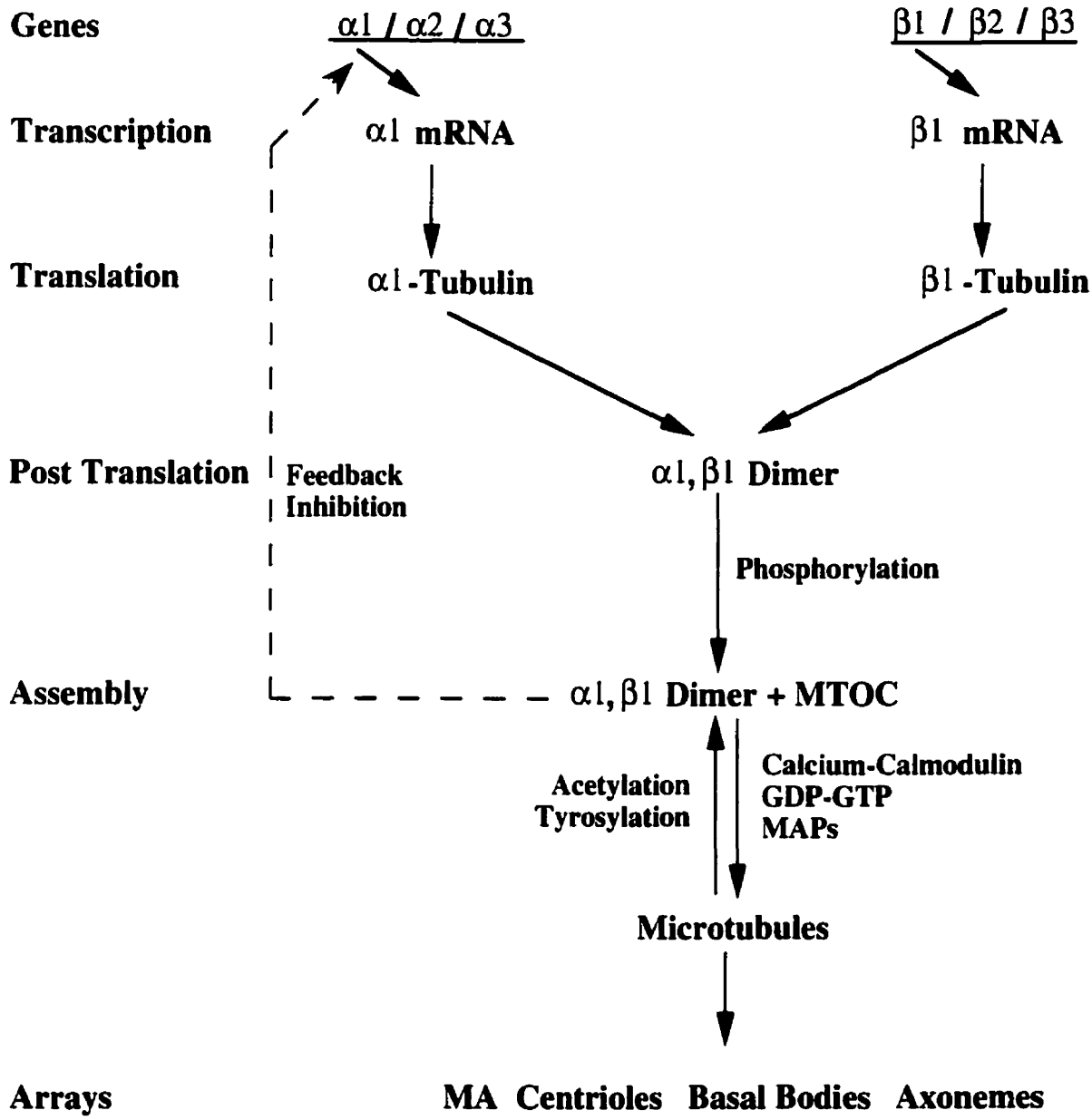
When microtubules are required by a cell for a particular function, microtubules assemble in the appropriate region of the cell, with the necessary orientation. As microtubules are no longer needed, they depolymerize. The word microtubules describes a class of similar

structures, formed of specific proteins called tubulins (5). Microtubules are comprised principally of dimeric subunits of one α- and one β-tubulin polypeptide and participate in a diverse spectrum of cellular functions including programmed modifications of cell shape during morphogenesis, formation of mitotic and meiotic spindles, and establishment of cilia and flagella-dependent cell motility, and organelle transport (Figure 1).

In different microtubules, the tubulins are copolymerized with any of a variety of proteins collectively known as microtubule-associated proteins, or MAPs, not to be confused with mitogen activated proteins (MAPs) which were themselves originally identified as microtubules associated proteins, hence the name. Both tubulins and certain groups of the MAPs may be coded by more than a single gene; both tubulins and MAPs are modified in various ways after they are synthesized, including phosphorylation and acetylation (27). Thus the determination of what sort of microtubule will be present at a particular time in a particular cell depends, at least in part, upon the synthesis and processing of a number of proteins. But the presence of the right proteins, while necessary, is not sufficient for assembly to occur.

The assembly of many, if not all microtubules depends on the presence and orientation of microtubule organizing centers (5), which may include a centriole or analogous structure. It is not clear however, what determines the length of a microtubule or what factors enable a cell to depolymerize its microtubules as required for normal cell function. In a living cell, one population of microtubules may be elongating at the same time as another population is deassembling.

Figure 1 Regulatory Pathway of Microtubule Assembly. This schematic outlines at least seven regulatory processes involved in tubulin synthesis and microtubule assembly. The microtubule organizing centre (MTOC) is a perinuclear apparatus composed primarily of γ-tubulin subunits (80) and is responsible for the nucleation of tubulin monomers into mature microtubule structures. MA - mitotic apparatus.


Genes
α1 / α2 / α3
β1 / β2 / β3
Transcription
α1 mRNA
β1 mRNA
Translation
α1-Tubulin
β1-Tubulin
Post Translation
Feedback Inhibition
α1, β1 Dimer
Phosphorylation
Assembly
α1, β1 Dimer + MTOC
Acetylation
Tyrosylation
Calcium-Calmodulin
GDP-GTP
MAPs
Microtubules
Arrays
MA Centrioles Basal Bodies Axonemes

2.2 Regulation of Microtubule Dynamics.

Microtubule integrity is regulated through a complex temporal and spatial process, due in part to its dynamic instability. A number of observations have demonstrated that changes in cell morphology have a direct influence on gene regulation (21,75), thus a defined feedback network must exist within the flux of cytoarchitecture and genetic expression. Cell cycle control of microtubules has been an extensively characterized process, involving differential requirements for microtubules during interphase and mitosis (114). Microtubules are also implicated in numerous other processes such as cellular differentiation of monocytic cells (83), specific adherence of cytotoxic T-cells to their respective target cell (55), and during endothelial cell migration to damaged tissue (61). All of these observations correlate with a concomitant change in gene expression. Depolymerization of microtubules with drugs such as colchicine and nocodazole have been shown to stimulate cellular proliferation in the absence of a primary signal (36), as well, a phorbol ester resistant U937 variant that is defective in microtubule reorganization, can be reconstituted to the normal phenotype upon addition of nocodazole (88). It has also been established that stabilization of microtubules can suppress the activity of mitogenic factors such as thrombin and epidermal growth factor (35) as well as interleukin 8 (160). Transcriptional activation has also been demonstrated for a number of genes including the urokinases type plasminogen activator (uPA) (26,98), interleukin-1β (49,108), and the integrin α chain, cd11b (88) are activated in the presence of microtubule depolymerizing agents such as nocodazole and cholchicine.

One potential mechanism by which microtubule dynamics may directly influence genetic regulation is through redistribution of various transcription factors which are resident in the cytoplasm and undergo translocation to the nucleus when exposed to various stimuli (73).

3. NF-κB - A cytoskeletally regulated family of transcription factors.

3.1 The NF-κB Family.

Recent evidence has established NF-κB as a cytoskeletally regulated transcription factor. NF-κB has been shown to be regulated by the small GTP-binding protein rac1 (135,166), an actin associated protein involved in actin reorganization. As well, NF-κB has also been shown to be activated upon depolymerization of microtubules with the antimitotic drug nocodazole (147).

NF-κB was first characterized as a nuclear protein which bound specifically to the κ light chain enhancer with specific activation in B cells (151) (for reviews see (15,16,59,171)). NF-κB constitutively binds to a decameric oligonucleotide with a consensus sequence consisting of 5'-GGGPuNNPyPyCC-3' (62). The binding symmetry of NF-κB appears to be rather distinct in its binding capacity. While other transcription factors also form heterodimers among their family members, each of the subunits have similar binding affinities and the DNA binding motifs are very specific. In the case of NF-κB there is considerable variation in the binding affinity of each subunit and the binding motifs tend to be slightly asymmetric, lending to the variability of NF-κB gene activation (180,181). NF-κB is comprised primarily of two major subunits: p50 and RelA (p65), which can adopt a heterodimer or homodimer conformation and thus alter the DNA binding specificity of each complex.

Common among the NF-κB family is a 300 amino acid homology domain referred to as the NRD (NF-κB/Rel/Dorsal). The NRD is the minimal domain required for DNA binding and shares its name with the Rel oncoprotein and the Drosophila factor dorsal, both of which contain NRD motifs (56,58,87,124). Structurally, the NRD is composed

of two domains, the N-terminal which represents the DNA binding domain (94) and the C-terminal portion which is important for dimerization of NF-κB subunits (104). Although p50 contains very little sequence information outside of the NRD, RelA, has a 250 a.a. C-terminal domain comprising two to three independent transactivation domains (116,154), suggesting that p65 is a necessary component of transcriptional activation by NF-κB. Following the initial discovery of p50 and RelA, other NRD possessing proteins were added to the NF-κB family. They are p105, the precursor protein of p50 (87,124), p100, a precursor of p52 (119), c-Rel (31,77), and RelB (150).

Subunit composition is an important factor establishing the activation potential of a particular complex (95,181). Figure 2 outlines the NF-κB family members, subdivided into subunits which express or lack a functional transactivation domain. The lack of a transactivation domain in p50 and p52 indicate that these subunits do not activate transcription and homodimers can behave as repressors, competing away RelA-containing heterodimers (119,133,154). Among those subunits with transactivation domains, the level of transactivation is quite variable. c-Rel has a much weaker transactivation potential than RelA, demonstrated by an attenuation of κB-dependent gene activation after overexpression of c-Rel (44). RelB is somewhat paradoxical; in murine cells RelB displays modest transactivation by dimerization with p50 but it does not bind to any of the known κB consensus sequences (150). Human RelB (I-Rel) appears to lack a functional transactivation domain and acts a suppressor of RelA-p50 heterodimers (149). Many of the studies to date have identified RelA, c-Rel, and p50 as the major NF-κB subunits that interact with the majority of κB consensus sites (16).

3.2 The IκB Family

NF-κB complexes are predominantly found as a latent cytoplasmic population

Figure 2 Schematic representations of the NF-κB family members. Each of the NF-κB subunit shares a highly conserved Rel homology domain in the N-terminal domain. depicted as  . The C-terminal region of the Rel homology domain houses a nuclear localization signal for nuclear transport called the NLS. The poly-G island represents a glycine hinge motif important for proteolytic processivity of p105 and p100 to generate p50 and p52 respectively. The C-terminal portion of p100 and p105 contain ankyrin repeat motifs depicted as  . Finally, some members of the NF-κB family also have a transactivation domain  in their C-terminal domain.

p100 (p52)

p105 (p50)

c-Rel

p65

Rel-B

complexed to the inhibitory protein IκB (14,20). The IκB interaction with NF-κB appears to serve two functions: (1) inhibition of NF-κB DNA binding, and (2) cytoplasmic retention of NF-κB. To date a number of IκB family members (Figure 3) have been described including; IκBα, IκBβ, IκBγ, IκBε, p105, p100, and bcl-3 (67,69,76,125,142,173,193).

All of the IκB related proteins share 5-7 copies of a repetitive 33 amino acid homology domain referred to as the ankyrin motif (67,76). These regions are homologous to the protein of the same name that functions as a cytoskeletally-associated protein by crosslinking the spectrin cytoskeleton of red blood cells to the plasma membrane (5). The ankyrin domain of IκBs interacts with the nuclear localization signal (NLS) of NF-κB dimers, effectively blocking nuclear transport of NF-κB (20,54) and its DNA binding potential (66,76). As well, IκBs also express an NLS that allows newly synthesized IκBs to translocate to the nucleus and effectively bind and disrupt NF-κB/DNA complexes, resulting in nuclear export of the resulting NF-κB/IκB complex to the cytoplasm (10,11,198).

The best characterized IκB subunit is IκBα (Figure 4). Activation of NF-κB depends on the inducible phosphorylation of IκBα on Ser-32 and Ser-36 leading to proteolytic degradation and release of the NF-κB complex (33,69,158,173). Degradation of IκBα is mediated by ubiquitination on lysine 21 and 22 in the signal response domain (SRD) (145,153) leading to *in situ* degradation by the 26S proteasome complex (128,177). Of interest, the ubiquitin pathway is also associated with the microtubule cytoskeleton (117,178). After stimulation with a various number of inducers (Table 1), IκBα is phosphorylated on serine 32 and 36 (Figure 4) (28,30,176). Since inducible IκBα degradation requires the SRD, it is interesting to note that basal turnover of IκBα appears to require the ankyrin repeat domain (92) yet both pathways are mediated by the same

Figure 3 Schematic representations of the IκB family members. All members of the IκB family share a highly conserved ankyrin repeat domain  . The precusor proteins, p100 and p105 both contain an IκB like domain which is important for regulating proteolytic cleavage of these precursor proteins. IκBγ is generated from the alternative splicing of p105 mRNA. Also present in IκBα and IκBβ is the PEST sequence region  , which has numerous proline, glutamic acid, serine and threonine residues important for the inherent stability of these proteins.

p100 (p52)

p105 (p50)

IκBγ

Bcl-3

IκBβ

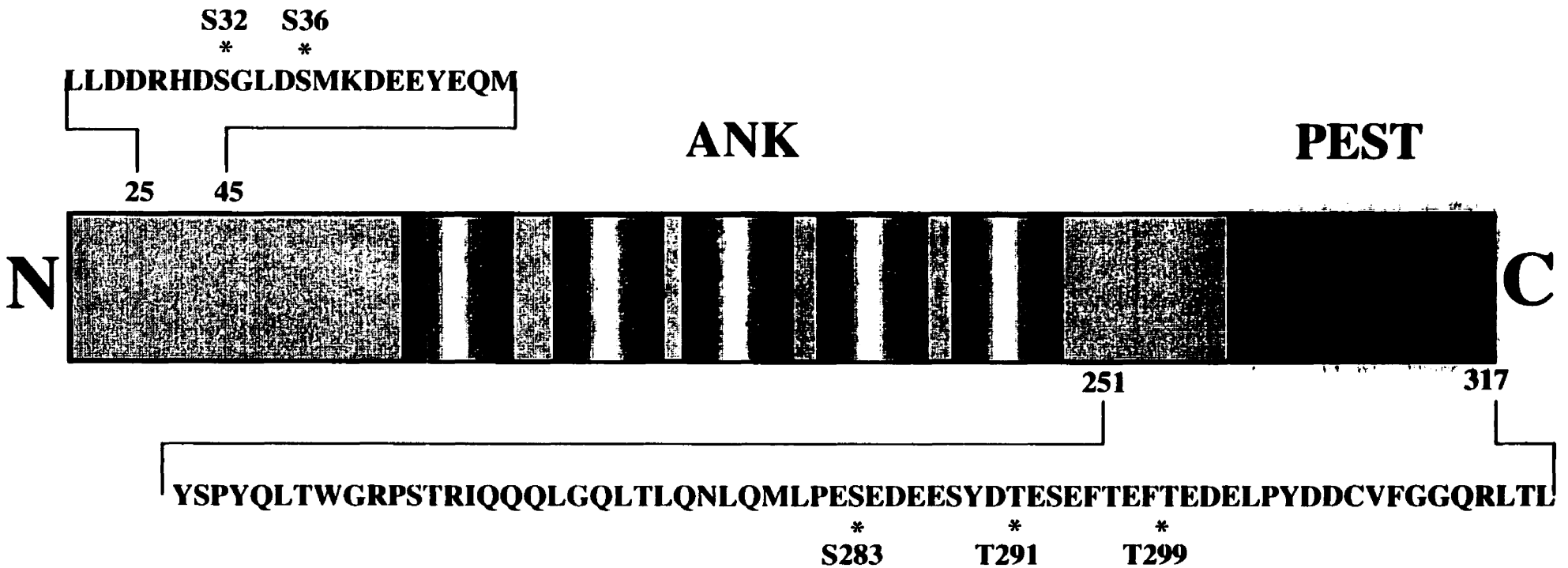
IκBα

proteasome complex. Furthermore, the intrinsic protein stability of IκBα is regulated by the C-terminal PEST domain (19,100). Point mutations in Ser-283, Thr-291, Thr-299, which results in loss of constitutive casein kinase II phosphorylation, increases the stability of IκBα, suggesting that C-terminal phosphorylation IκBα may also be important for its degradation. In relation to these observations, protein chimeras containing the N-terminal domain of IκBα, exhibit inducible phosphorylation (29). Addition of the C-terminal domain results in both phosphorylation and degradation of the chimera, reinforcing the importance of the IκBα C-terminal domain in inducible proteolysis. In addition, tyrosine phosphorylation at Tyr-42 negatively regulates inducible phosphorylation and degradation of IκBα (162). New evidence also demonstrates that phosphorylation of p65 on Ser-276 is an important step in transactivation of NF-κB (202). This process is mediated by the IκB-associated PKA catalytic subunit and might allow for control of NF-κB transcription levels through modulation of p65 phosphorylation.

3.2.1 The IκB Kinase

Since the mechanism of phosphorylation of IκB was first characterized several laboratories sought to characterize an IκB kinase complex that directly mediates inducible phosphorylation of IκB family members. Recently, a number of groups have characterized a 900 kD protein complex that contains the IkB kinase activity (IKK/CHUK) (113,139,194,199) comprised of two serine kinases IKKα and IKKβ (for review see (164)). The two subunits have an identity of 52% and contain an N-terminal catalytic domain as well as a leucine zipper and helix-loop-helix motifs in their C-terminal domain that may play a role in dimerization of the two subunits. In order to characterize IKK, the NF-κB inducing kinase NIK whose homology matches the MAP kinase kinase

Figure 4 Schematic of IκBα. Human IκBα has 5 ankyrin repeats, which represent the interacting domains for NF-κB subunits. The N-terminal region between amino acids 25-45 contains the signal response domain (SRD) of IκBα and has been expanded in the diagram to highlight two amino acid residues. namely serine 32 (S32) and serine 36 (S36) (asterisks). Following induction with a number of stimuli, both S32 and S36 are phosphorylated, leading to the eventual ubiquitination and degradation of IκBα. As well, the PEST domain, spans the region between 264 and 317 and belongs to a region of IκBα (residues 251-317) that has a number of casein kinase II phosphorylation sites including serine 283 (S283), threonine 291 (T291), and threonine 299 (T299) as denoted by the asterisks (100).

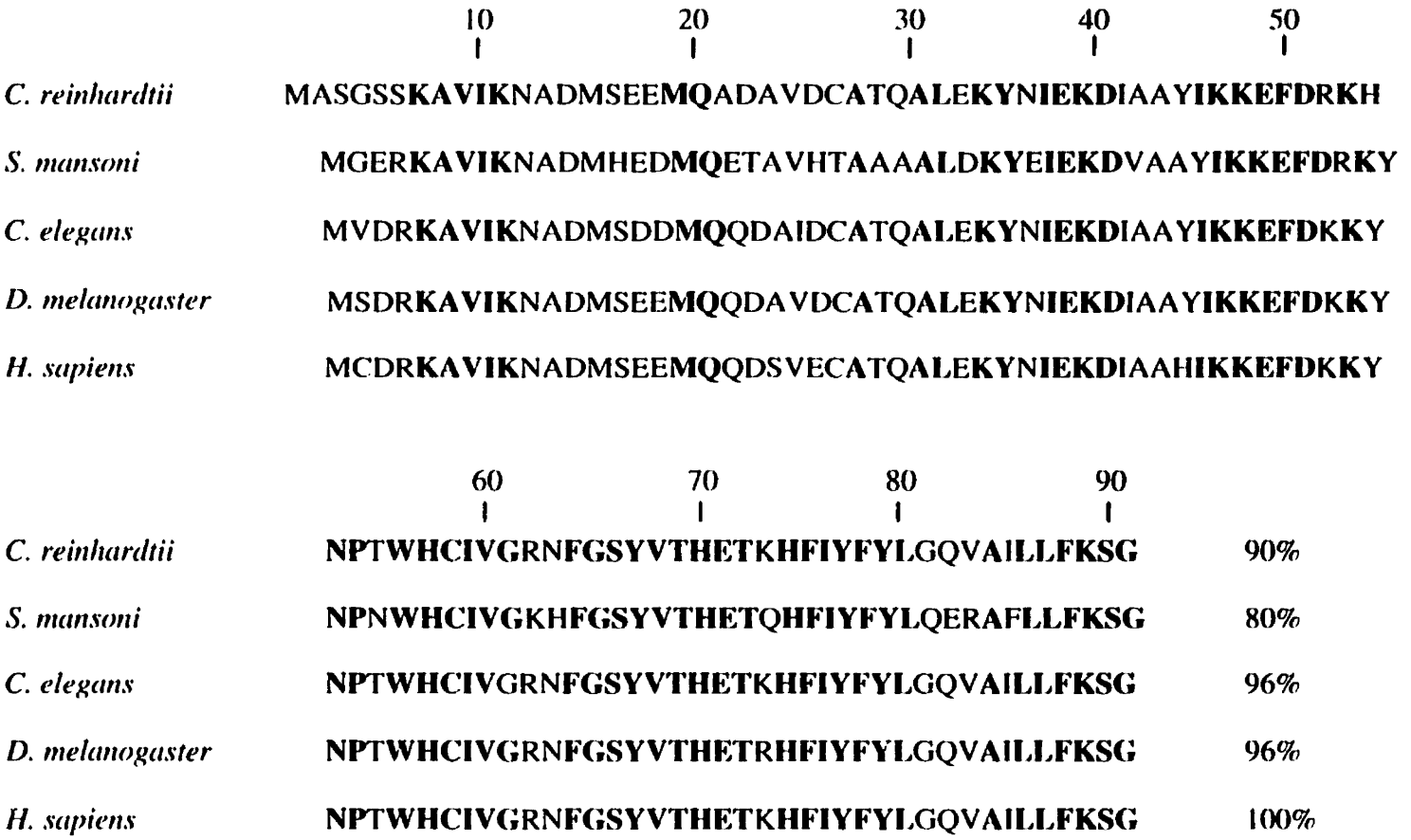

S32
S36
*
*
LLDDRHDSGLDSMKDEEYEQM
ANK
PEST
25
45
N
C
251
317
YSPYQLTWGRPSTRIQQQLGQLTLQNLQMLPESEDEESYDTESEFTEFTEDELPYDDCVFGGQRLTL
*
*
*
S283
T291
T299

kinase (MAP3K) and represents a common check-point in IL-1 and TNF-α signaling (106) was used to identify IKK (139). *In vitro* kinase assays using purified IKKα and IKKβ demonstrated that IKKα was responsible for phosphorylating Ser-32 and 36 on IκBα (113,139,194,199) while IKKβ appeared to display a more potent activity in the phosphorylation of IκBβ at Ser-19 and 23 (194), the inducible phosphorylation sites of IκBβ (191). Presently, the IKK complex is activated in the presence of TNF-α, IL-1, and PMA (113,199), and its activation appears to be sensitive to the phosphatase PP2A, suggesting that phosphorylation by NIK may control its activity. It is not clear however whether IKK directly phosphorylates IκB *in vivo* since all IKK data was obtained from overexpression assays or *in vitro* translation systems. Rather, IKK may belong to a complex responsible for activation of a true IκB kinase (164).

3.2.2 IκBα as a microtubule-associated protein

Recent evidence has shown that the signal response domain of IκBα interacts with the dynein light chain (DLC) subunit of the cytoplasmic dynein motor protein complex (34). DLC was first characterized as an 8 kDa component of the outer arm dynein of *Chlamydomonas* (91), its cytoplasmic counterpart being highly conserved among a number of higher eukaryotes including plants, drosophila, nematodes, and humans (Figure 5). The function of dynein light chains is still unknown but a number of groups have made observations that shed new light on this relatively new member of the dynein motor complex. Work in *Drosophila melanogaster* has established that mutations in DLC (ddlc1) produce a variety of morphogenetic defects including embryo lethality displaying DNA degradation and membrane blebbing characteristic of apoptosis (42). The mouse dynein light chain (Tctex-1) has been implicated in the transmission ratio distortion (meiotic drive) during meiosis (90). In normal mice, the *t*-complex of chromosome 17

Figure 5 Sequence homology of DLC-1 between various species. Identical amino acid residues are shown in bold type. Homology to human DLC-1 is shown. DLC-1 is a 91 amino acid protein with an apparent molecular weight of 9kDa.


10 20 30 40 50
C. reinhardtii MASGSSKAVIKNADMSEEMQADAVDCATQALEKYNIEKDIAAYIKKEFDRKH
S. mansoni MGERKAVIKNADMHEDMQETAVHTAAAALDKYEIEKDVAAYIKKEFDRKY
C. elegans MVDRKAVIKNADMSDDMQQDAIDCATQALEKYNIEKDIAAYIKKEFDKKY
D. melanogaster MSDRKAVIKNADMSEEMQQDAVDCATQALEKYNIEKDIAAYIKKEFDKKY
H. sapiens MCDRKAVIKNADMSEEMQQDSVECATQALEKYNIEKDIAAHIKKEFDKKY
60 70 80 90
C. reinhardtii NPTWHCIVGRNFGSYVTHETKHFIYFYLGQVAILLFKSG 90%
S. mansoni NPNWHCIVGKHFGSYVTHETQHFIYFYLQERAFLLFKSG 80%
C. elegans NPTWHCIVGRNFGSYVTHETKHFIYFYLGQVAILLFKSG 96%
D. melanogaster NPTWHCIVGRNFGSYVTHETRHFIYFYLGQVAILLFKSG 96%
H. sapiens NPTWHCIVGRNFGSYVTHETKHFIYFYLGQVAILLFKSG 100%

which contains several sequence inversions and also encodes for Tctex, is important for suppression of recombination during meiosis. This allows for *t*-haplotypes to be inherited as a single unit. In cases where two complementary *t*-haplotypes (homozygosity) are generated, male mice are completely sterile, suggesting a role for cytoplasmic dynein dysfunction in non-mendelian chromosome segregation (90).

Cytoplasmic dynein is a large multi-subunit complex (129) first identified as a microtubule associated protein (MAP 1C) which translocated along microtubules in a direction opposite that of the anterograde kinesin motor complex (130). Dynein is comprised primarily of two catalytic heavy chains (532 kD), several intermediate chains (74 kD), light intermediate chains (53-59 kD), and light chains (8-24 kD) (184). Cytoplasmic dynein is a multi-functional, cell cycle regulated complex involved in retrograde transport of vesicles (43,121), maintenance of the Golgi complex (182), and spindle pole formation during mitosis (for reviews see (8,18,47,183,189,195)). The major regulatory subunit of dynein motility is the dynactin complex which physically links cytoplasmic dynein to a particular carrier species (4,57,152,157,187). One curiosity of dynein is that its cargo specificity is regulated by casein kinase II (82), the same kinase which regulates the intrinsic stability of IκBα (100), suggesting that casein kinase II may play a role in the regulation of IκBα/Dlc-1 interactions.

A yeast two-hybrid screen was used to isolate DLC-1 cDNA. Overexpression of DLC in L40 yeast demonstrated a strong interaction with the N-terminal domain of IκBα (Figure 6). As well, DLC-1 did not compete for p65 binding to IκBα, suggesting they contact the molecule at different domains *in vitro*. To verify that IκBα and DLC-1 interacted in mammalian systems, immunoprecipitation of *in vitro* purified DLC-1-GST fusion proteins were incubated with whole cell extracts containing IκBα (Figure 7A). *In vivo* immunoprecipitations were carried out with a DLC-1-myc fusion protein overexpressed

Figure 6 DLC-1 Interacts with the signal response domain of IκBα. Five IκBα chimeras were fused downstream of the LexA DNA binding domain (NIK and NIK2N) or the Gal4 transactivation domain (IκBα, ANK, CIK). Of the chimeras tested, only full length IκBα, NIK, and NIK2N interacted with DLC-1 but not ANK or CIK. DLC-1 expressing yeasts were mated to the AMR70 strain of yeast expressing the different chimeras. The color of the colonies is indicative of β-galactosidase activity. Blue indicates a positive interaction (color detectable within 30 min), and white colonies indicate no interaction (incubation time >16 h).

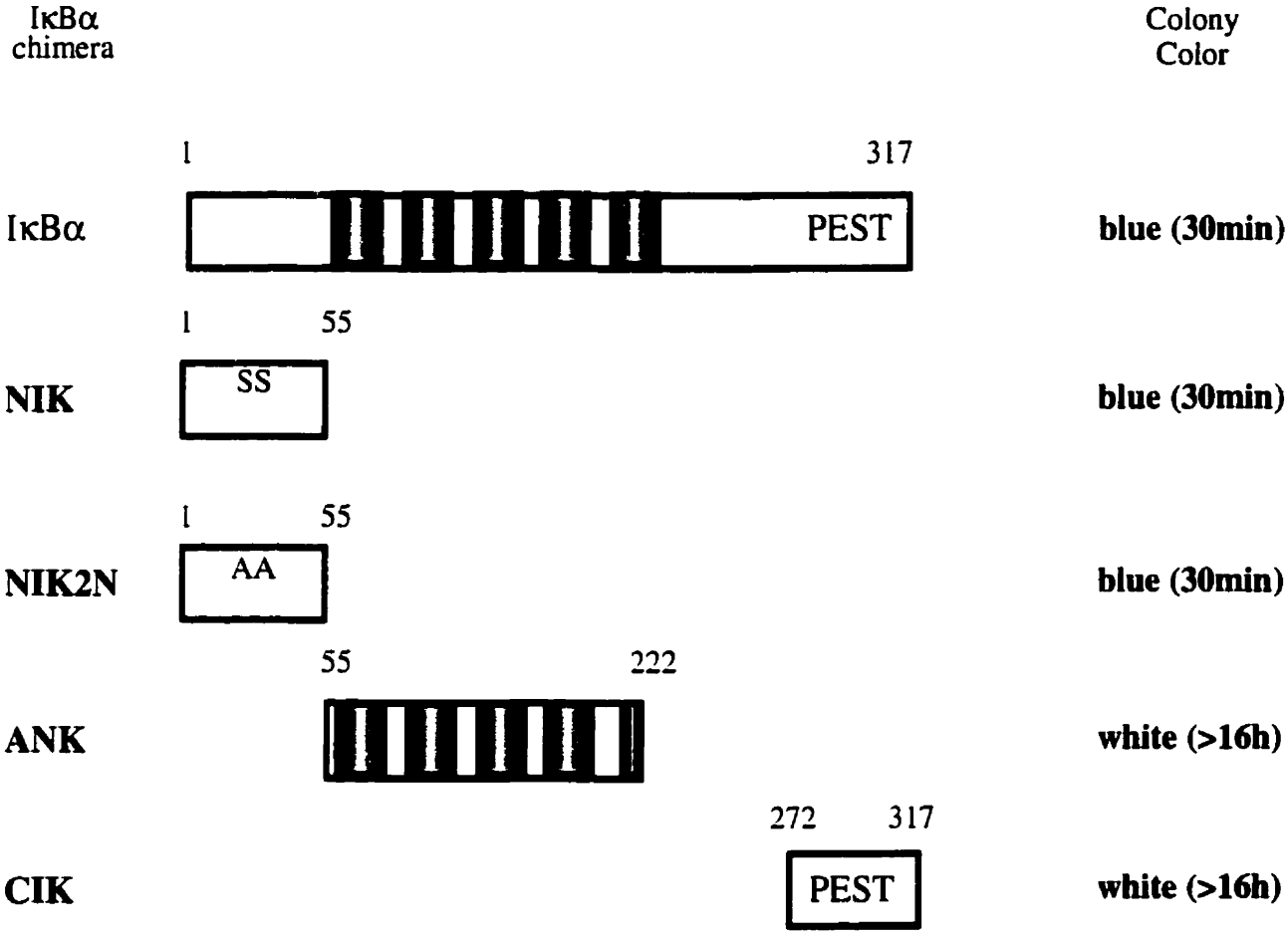
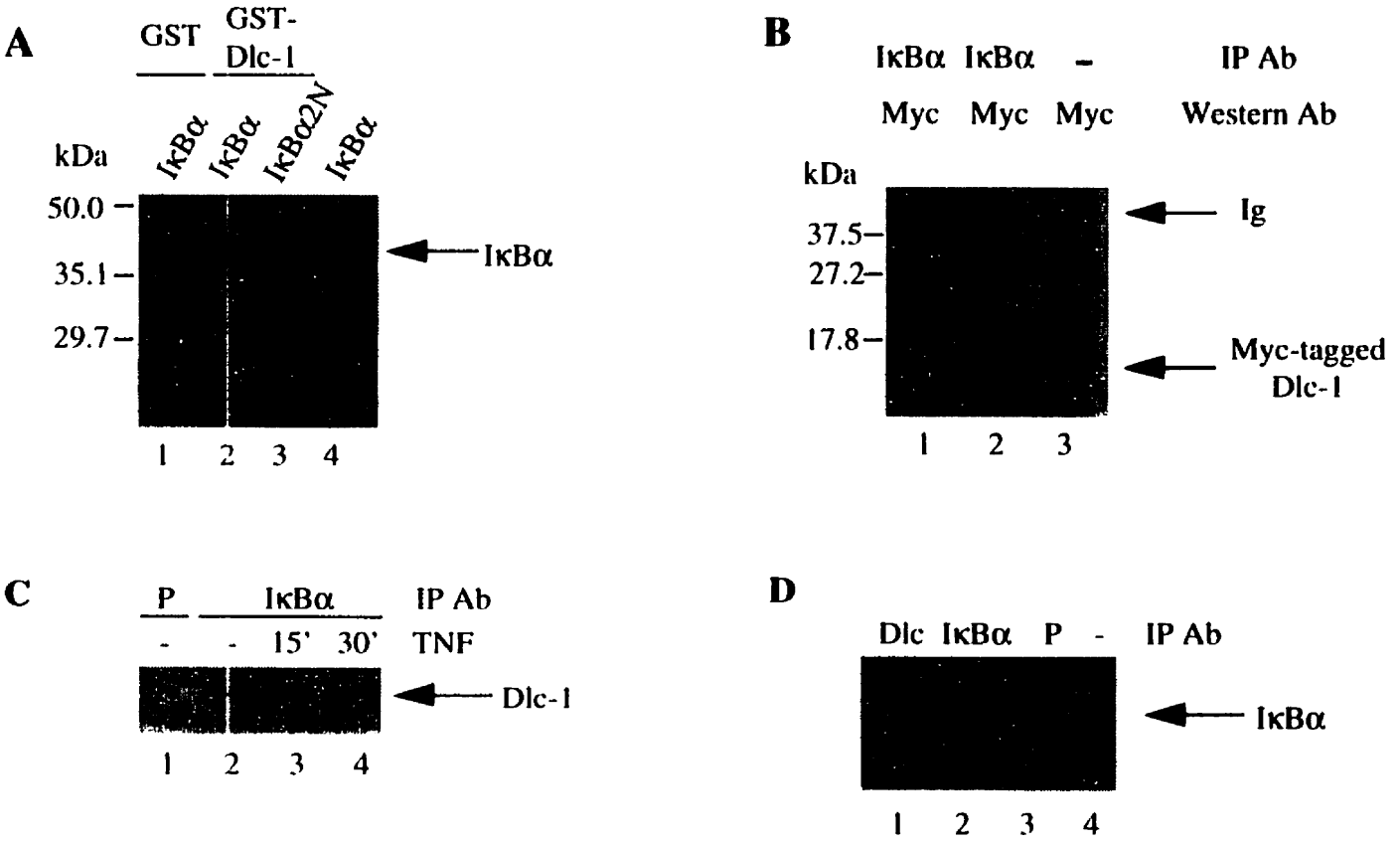

IκBα chimera
Colony Color
1
317
IκBα
PEST
blue (30min)
1
55
NIK
SS
blue (30min)
1
55
NIK2N
AA
blue (30min)
55
222
ANK
white (>16h)
272
317
CIK
PEST
white (>16h)

Figure 7 IκBα **interacts with DLC-1 in vitro and in vivo.** (A) In vitro interaction of DLC-1 and IκBα. GST (lane 1) and GST-DLC-1 (lanes 2 and 3) were incubated in the presence of IκBα (lanes 1 and 2) or IκBα2N (lane 3). Lane 4 shows crude bacterial extract of IκBα protein as migration control. Western blot analysis was performed using the AR20 IκBα antibody (9). (B) In vivo interaction of DLC-1 with IκBα. Lysates of HeLa cells transfected (lanes 2 and 3) with Myc-tagged DLC-1 or untransfected (lane 1) were immunoprecipitated with the anti IκBα antibody (lanes 1 and 2). Lane 3 outlines migration of crude transfected HeLa extracts as a control. The anti-Myc antibody was used for western blot analysis. (C) In vivo interaction of DLC-1 and IκBα. 293 cells were transfected with IκBα and Myc-tagged DLC-1 (10 μg each); at 48 hours post-transfection, cells were treated with 10ng/ml TNF-α for 15 and 30 minutes to degrade IκBα. Whole cell extracts were immunoprecipitated with a C-terminal IκBα antibody (Santa Cruz, Inc.) and analyzed for DLC-1 using the anti-Myc antibody: P, preimmune serum. (D) In vivo interaction of DLC-1 and IκBα. Anti-GST-DLC-1 antibody was used to immunopreciptate endogenous DLC-1 complexes from 293 cell lysates (1 to 3 mg; immune complexes were then isolated on protein A-sepharose and analyzed for IκBα by western blot with anti-N-terminal IκBα (Santa Cruz, Inc.). Whole cell extracts were loaded as controls; P, preimmune serum.


A
GST
GST-Dlc-1
IκBα
IκBα
IκBα2N
IκBα
kDa
50.0
35.1
29.7
IκBα
1 2 3 4
B
IκBα IκBα -
IP Ab
Myc Myc Myc
Western Ab
kDa
37.5
27.2
17.8
Ig
Myc-tagged Dlc-1
1 2 3
C
P
IκBα
IP Ab
- - 15' 30'
TNF
Dlc-1
1 2 3 4
D
Dlc IκBα P -
IP Ab
IκBα
1 2 3 4

in HeLa cells (Figure 7B). In both cases, IκBα was successfully immunoprecipitated establishing that DLC-1 interacts with IκBα in mammalian cells. Further immunoprecipitations also demonstrated that endogenous DLC-1 interacted with IκBα (Figure 7C) and that pretreatment with TNF-α (Figure 7D) was sufficient to disrupt this interaction establishing the specificity of IκBα and DLC-1.

Indirect immunofluorescence of IκBα and DLC-1 indicate that IκBα and DlC-1 colocalize to the microtubule organizing centre (MTOC) along the microtubule matrix. As well, p65 was also shown to colocalize with DLC-1 and IκBα. The evidence suggests that IκBα and NF-κB exist as a latent cytoplasmic complex through the association of IκBα with DLC-1 at the MTOC and establishes a cytoplasmic retention model for NF-κB regulation. NF-κB is not the only transcription factor that is retained in this manner. Recently, the kinesin superfamily motor KIF3 was shown to interact with the MAPKKK MLK2 (mixed lineage kinase) and co-localized with activated JNK along microtubules (118). More specifically, MLK2 interacts with the KAP3A targeting component of KIF3, highlighting an intriguing association between stress activation and motor protein regulation.

3.3 Signal-induced regulation of NF-κB transactivation

Numerous transduction pathways are implicated in NF-κB signaling as are the conditions involved in NF-κB activation (Table 1). In most cases, NF-κB-dependent gene activation relies on regulatory signals that induce IκB phosphorylation (16). Activation of NF-κB with double stranded RNA requires the double-stranded RNA-dependent protein kinase (PKR) which is capable of directly phosphorylating IκBα at Ser-32 and 36 (93). TNF-α stimulation of NF-κB requires activation of various TNF receptors (TNFRs) followed

Table 1 Inducers of NF-κB Transactivation

Bacterial Products	Lipopolysaccharide
	Exotoxin
	Muramyl peptides
Viruses	Human immunodeficiency virus (HIV-1)
	Human T-cell leukemia Virus (HTLV-1)
	Hepatitis B Virus (HBV)
	Herpes simplex virus (HSV-1)
	Epstein-Barr Virus (EBV)
	Adenovirus
Viral Products	
	Double-stranded RNA
	Tax (HTLV-1)
	Tat (HIV-1)
	Hbx (HBV)
	MHBs (HBV)
	EBNA-2 (EBV)
Inflammatory cytokines	Tumor Necrosis Factor alpha
	Lymphotoxin
	Interleukin-1
	Interleukin-2
	Leukotriene B4
T cell mitogens	Antigen
	Lectins (PHA, ConA)
	Calcium ionophores\
	Anti CD28
Physical stress	UV light
	γ radiation
Protein synthesis inhibitors	Cyclohexamide
	Anisomycin
Drugs	Okadaic acid
	Phorbol esters

Adapted from (16)

by downstream signaling through the TNF receptor associated factor 2 (TRAF2) (106,164). Just as TNF-α signals through TRAF2, IL-1 uses a similar receptor-mediated system to activate NF-κB, including TRAF6 and the IL-1 receptor activated kinase (IRAK) (2) suggesting both pathways may share common signaling elements leading to NF-κB activation.

3.3.1 Phorbol Esters Activate NF-κB

Distinct from TNF-α and IL-1 signaling is the phorbol ester signaling pathway. Phorbol esters such as phorbol, 12-myristate, 13-acetate (PMA) derive their activating potential by mimicking diacylglycerol (DAG), a common biproduct of phosphotidylinositol breakdown (71). Both of these compounds are capable of directly activating the lipid-inducible protein kinase C (PKC) (for review see (71,120)). Activated PKC is capable of phosphorylating IκBα directly (85,101), in many cases, PMA stimulation results in the activation of a number of PKC isoforms, of which, two appear to be important in the NF-κB pathway, namely, PKC-α and PKC-ζ (41,50,74,110,155).

Interestingly, prolonged exposure to phorbol esters leads to suppression of PKC activity through depletion of phorbol ester-responsive PKC isoforms (196). Depletion of PKC involves a ubiquitin-dependent covalent modification, followed by proteasome-mediated degradation of PKC (105). Besides the direct involvement of PKC in phosphorylation of IκBs, NF-κB activation has also been demonstrated in a PKC-mediated MAPK-dependent pathway (159). Phorbol-activated PKC is able to activate the MAP kinases ERK1/2 through direct activation of the c-Raf oncoprotein (3,32). Following c-Raf activation, the MAPK/ERK kinase (MEK) is activated, in turn phosphorylating and activating ERK1/2 cascade (6,132,200). Of the many ERK substrates, one, important for NF-κB activation, is the p90 ribosomal S6 kinase ($pp90^{rsk}$ or RSK) (156). RSK (see

review, (25)) was shown to be directly phosphorylated by ERK1/2 (22,165). Activated RSK subsequently interacts with IκBα and phosphorylates residue 32 (156). This demonstrates that RSK is not sufficient to induce IκBα degradation alone and may require another phorbol ester-regulated kinase, such as IKK, to contribute to serine 36 phosphorylation. However, transdominant mutants of RSK resulted in the complete abrogation of NF-κB activation in the presence of phorbol ester, providing evidence for the requirement of multiple IκBα kinases for induction of IκBα degradation.

3.4 NF-κB regulates numerous genes

Inducible degradation of IκBs exposes the nuclear localization signal of the NF-κB dimers allowing for rapid nuclear transport of NF-κB subunits and activation of NF-κB-specific genes (see Table 2). NF-κB is not itself sufficient for transcriptional activation of the basal complex and requires a number of other subunits to facilitate activation. Characterization of the IFNβ promoter has revealed that the enhanceosome is a multi-subunit complex consisting of p50/p65 NF-κB subunits, the interferon regulatory factor 1 (IRF-1), activated T-cell factor-2 (ATF-2), the JNK-inducible c-Jun transcription factor, and HMG-I(Y) (45,48). The high mobility group protein [HMG-I(Y)] is required to bend the enhancer region DNA to facilitate association of the enhanceosome with the basal transcription machinery (172).

Of the genes regulated by NF-κB, IκBα is a major transcriptional target, representing a novel autoregulatory loop (38,97). However, IκBβ is not regulated transcriptionally by NF-κB and is responsible for the persistent activation of NF-κB by LPS and IL-1 (167,173). The inducibility of NF-κB can be modulated depending on the type of

Table 2 - Genes activated by NF-κB.

Viruses	Human immunodeficiency virus 1 (HIV-1) Cytomegalovirus Adenovirus Simian virus 40
Immunoreceptors	Immunoglobulin κ light chain T cell receptor β T cell receptor α β_2- microglobulin
Cell adhesion molecules	Endothelial leukocyte adhesion molecule 1 Vascular cell adhesion molecule 1 Intercellular cell adhesion molecule 1
Cytokines and hematopoietic growth factors	Interferon-β Granulocyte/macrophage colony-stimulating factor Interleukin 2 Interleukin 6 Interleukin 8 TNF-α Lymphotoxin
Acute phase proteins	Angiotensin Serum amyloid A percursor Urokinase-type plasminogen activator
Transcription factors and subunits	c-rel NF-κB precursor p105 c-myc Interferon regulatory factor 1
Others	Vimentin cytoskeletal protein NO-synthase

Adapted from (16)

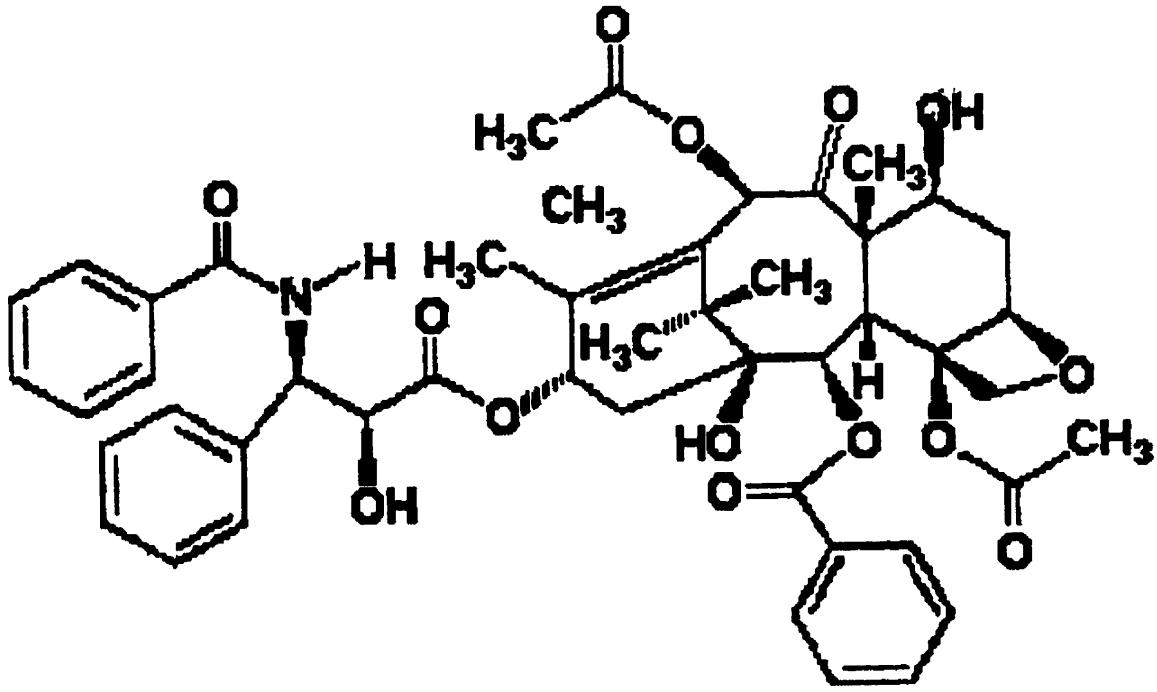
inducer. For instance, TNF-α and PMA rapidly induce NF-κB activation (1), whereas IL-1 and LPS display slower activation kinetics but maintain persistent transactivation. NF-κB regulation is also important for a number of viral genomes including the retroviruses HIV-1 and HTLV (for review see (148)). The proviral genomes contain NF-κB consensus sequences in the U3 region of the LTR and modulate NF-κB activity by viral factors such as Tax (HTLV-I) (16), and Tat (HIV-1).

4. Taxol: A microtubule stabilizing agent

Taxol was first isolated from the pacific yew *Taxus brevifola* in the early 70's (190), displaying a novel antitumor activity. Structurally, taxol is a diterpenoid (Figure 8) which can be obtained from either the bark of yew trees or from chemical synthesis (122). Since the chemical synthesis of taxol was attained, a number of other taxoid compounds have been generated. The taxol family is unique among microtubule stabilizing agents in that it is capable of stabilizing microtubules against depolymerization through interaction with the amino-terminal region of β-tubulin (138).

A unique characteristic of taxol is that its therapeutic effectiveness is much greater than that of other known microtubule disrupting compounds such as colchicine and nocodazole, suggesting taxol may display secondary effects beyond microtubule stability and may exert direct effects on signal transduction pathways (99). Taxol behaves as an antimitotic drug which blocks cells in the G2/M phase by preventing the breakdown and reassembly of the mitotic spindle apparatus (12). Prolonged exposure of cells to taxol results in programmed cell death or apoptosis (63) . It is believed that taxol induces apoptosis by facilitating phosphorylation of the anti-apoptotic factor bcl-2 (64), which is accompanied by the loss of bcl-2 function (65) and requires activated c-Raf (24).

Figure 8. Chemical structure of Taxol. Taxol was initially isolated from the bark of the pacific yew (*Taxus brevifolia Nutt*) in 1971. Taxol is comprised of a side chain, critical for maintaining activity as well as the taxol ring which consists of several rings: a four membered ring, a six membered ring, and an eight membered ring, as well as a number of peripheral functional groups. Chemical characterization suggests that the eight membered ring can be effectively contracted to a seven member ring without effecting its microtubule stabilizing potential.


O
H3C
O
O
OH
CH3
CH3
O
H
N
H3C
CH3
H3C
O
O
H
O
HO
O
O
CH3
O
OH
O
O

However, taxol-activated c-Raf is distinct from the PMA-activating c-Raf suggesting a novel c-Raf signaling pathway. Furthermore, bcl-2 has been described as the 'guardian of microtubule integrity' since drugs which disrupt normal microtubule function have been correlated with loss of bcl-2 anti-apoptotic function and may represent a normal physiological pathway for the elimination of cells with damaged mitotic apparatus (63).

Research Objectives

The objective of this thesis is to determine what regulatory effect taxol has on NF-κB activation through its ability to stabilize the microtubule cytoskeleton. This project will focus on NF-κB dependent gene expression and DNA binding including inducibility of IκBα phosphorylation and degradation. As well, cytological studies will convey to what extent various inducers of NF-κB including TNF-α, PMA, and LPS have on the cytoarchitecture of microtubules and whether this can be correlated with the outcome of NF-κB activity. Furthermore, attempts will be made to identify any taxol-sensitive elements (ie kinases) whose activity is crucial for NF-κB regulation.

METHODS AND MATERIALS

1. Cell Culture

Murine NIH 3T3 fibroblasts (ATCC) and COS-7 cells were maintained in Dulbeco's Modified Minimal Medium (DMEM) (GIBCO, Life Technologies Inc., Grand Island, N.Y.) supplemented with 5% calf serum (3T3) or 10% Fetal Bovine Serum (HeLa), 2mM L-glutamine, and 10μg of gentamicin. Human embryonic kidney cells (293) were maintained in Alpha Modified Minimal Medium (α-MEM) (GIBCO) supplemented with 10% Fetal Bovine Serum, 2mM L-glutamine, and 10μg gentamicin. All cells were maintained in a 37°C incubator with 5% CO_2.

2. Drug Induction Procedure

Exponentially growing cells representing a confluency of roughly 80% were treated with various drugs according to each protocol: tumor necrosis factor (TNF-α) at a final concentration of 10 ng/ml (Sigma); phorbol, 12-myristate, 13-acetate (PMA) at 100 ng/ml (Sigma), lipopolysaccharide (LPS) at 100 ng/ml (Sigma), Nocodazole at 20 μM (ICN), taxol at 5 μM (ICN). Cells were pre-incubated with taxol 30 min. prior to induction with the various compounds. For analysis of IκBα turnover, 50 μg/ml cylcohexamide (Sigma) was used to prevent resynthesis of IκBα.

3. Plasmids and Reagents

PMA (stored at 100 μg/ml in ethanol), TNF (100 μg/ml in ddH_2O), LPS (100 μg/ml in ddH_2O), Nocodazole (10 mM in DMSO), were purchased from Sigma Chemical Co. (St. Louis, MO). Taxol (5mM in DMSO) was purchased from ICN Pharmaceuticals, Inc (Costa Mesa, CA). The HIV enhancer CAT containing double repeats of the NF-κB consensus sequence (GGGACTTTCC) and the HIV enhancer mutant which is mutated in

both consensus regions (CATGGTTTCC) were used for determination of NF-κB specific gene activation.

4. Preparation of Protein Extracts

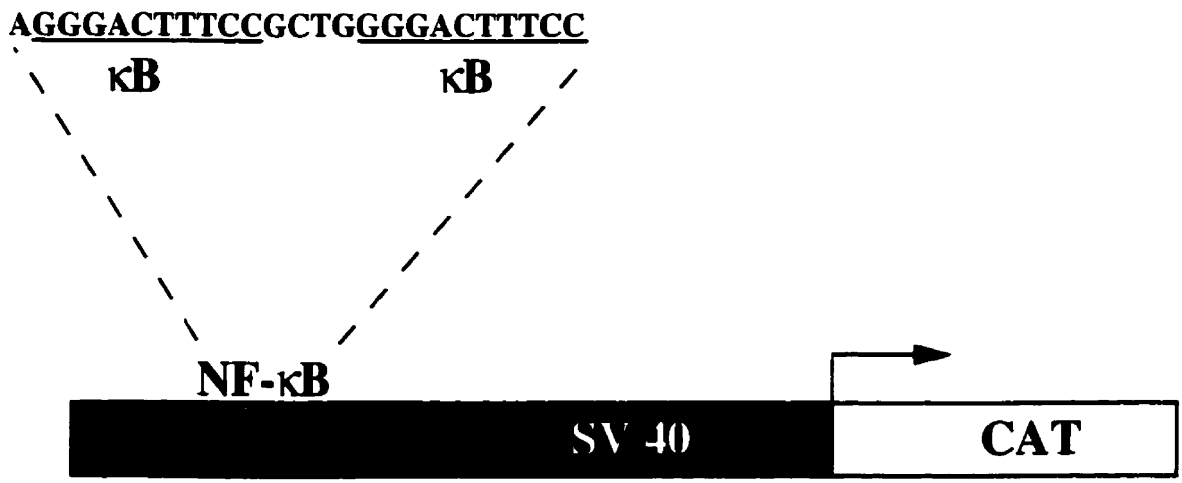
4.1 Nuclear and Cytoplasmic Extracts

Differential extraction of nuclear proteins from NIH 3T3 and 293 cells were accomplished using a previously reported protocol (127) with the following modifications. Briefly, cells were collected by centrifugation (10,000g) and washed once in PBS, and once in Buffer A (10 mM Hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes) pH 7.9; 1.5mM $MgCl_2$; 10 mM Kcl; 0.5 mM Dithiothreitol (DTT) and 0.5 mM phenylmethyl sulfonyl flouride (PMSF)) and were resuspended in 20 µl of Buffer A containing 0.1% NP-40. Cells were incubated on ice for 10 minutes and centrifuged at 10,000 g for 10 minutes. The supernatant (cytoplasmic fraction) was collected and pellets (nuclei) were resupended in 15 µl Buffer B (20 mM Hepes pH 7.9; 25% glycerol; 0.2 mM EDTA; 50mM KCl; 0.5 mM DTT; 0.5mM PMSF; 0.01 mg/ml Leupeptin; 0.01 mg/ml Pepstatin; 0.01 mg/ml Aprotinin; 0.01 mg/ml Spermidine and 0.01 mg/ml Spermine). Cells were stored on ice for 15 min and collected by centrifugation. Protein concentration was determined by Bradford protein assay (Bio-Rad).

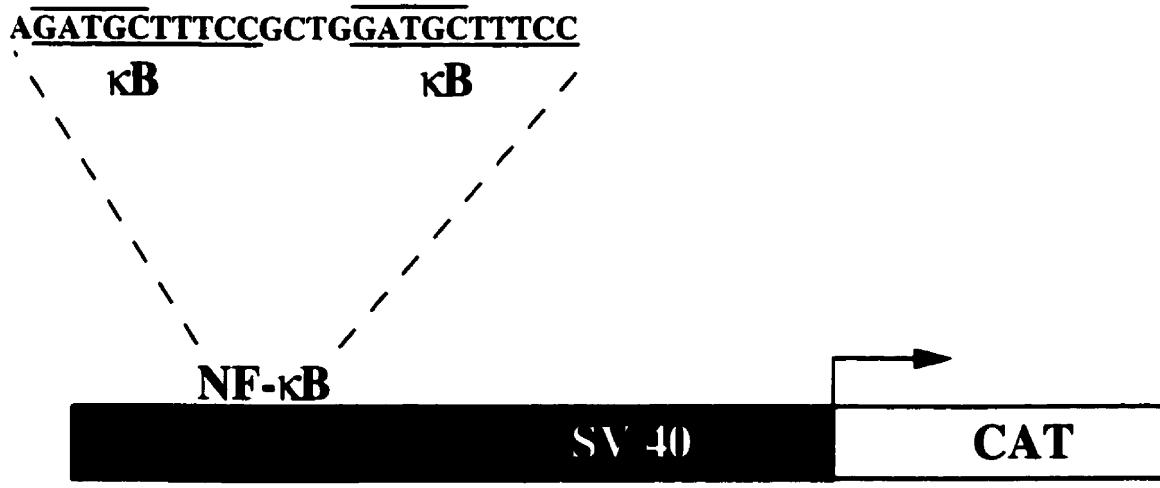
4.2 Whole Cell Extracts

After treatment with various drugs, cells were washed once with PBS and incubated for five minutes with 1 mL TEN buffer (10 mM Tris-HCL pH 7.5; 1 mM EDTA; 60 mM NaCl) to lift cells from plates. Cells were collected and centrifuged for 2 minutes at 10,000 rpm. Cells were washed once with PBS and resuspended in lysis buffer

Figure 9. Schematic representation of the pHIVenhCAT. The upstream promoter elements of the the pHIVenh-CAT and pHIVenh-mut-CAT plasmid are shown. The pHIVenh-CAT contains a single copy of the HIV-1 enhancer region (-105 to -80) which houses two κB consensus binding sequences (▲▲) linked to the basal SV40 promoter (SV 40). The pHIVenh-mut-CAT is similar to pHIVenh-CAT except that mutations in the κB consensus (overscored line) have been introduced to disrupt binding of NF-κB.



pHIVenh-CAT



pHIVenh mutant-CAT

(10 mM Tris-HCL pH 8.0; 1 mM EDTA; 60 mM: 1 mM DTT: 0.5% NP-40; 0.5 mM PMSF: 0.01 mg/ml Leupeptin; 0.01 mg/ml Pepstatin; 0.01 mg/ml Aprotinin). Cells were incubated for 15 minutes on ice and centrifuged at 10,000 g for 10 minutes. Supernatants were quantified for protein concentration using Bradford Reagent (Bio-Rad).

5. Electromobility Shift Assay (EMSA)

Nuclear extracts (5μg) were diluted to a total volume of 15 μl with DNA binding buffer (20 mM Hepes pH 7.9: 5% glycerol: 0.1 M KCl: 0.2 mM EDTA pH 8.0: 0.2mM EGTA pH 8.0). Reactions were pre-incubated for 10 min. at room temperature with the non-specific DNA competitor poly (dI:dC) (Pharmacia). Binding activity was analyzed using a [γ–^{32}P]-labeled probe corresponding to the PRD II domain of the IFN-β promoter (5'-GGGAAATTCCGGGAAATTCC-3') according to previously reported protocols (146). Protein extracts were incubated with 0.2 ng of probe corresponding to approximately 100.000 CPM/μl for 20 min. at room temperature. Resulting protein-DNA complexes were resolved by non-denaturing gel electrophoresis on a native tris-glycine polyacrylamide gel (5%: 60:1 crosslinker) and revealed by autoradiography. In order to facilitate specificity of the DNA-protein complexes. 125M excess of cold unlabeled probe was used as a competitor. For super shift analysis. rabbit polycolonal antibody (1 μl) directed against human *c-Rel*. p105/p50. or p65 (136) were incubated with nuclear extracts during the poly (dI:dC) step prior to the addition of labelled probe. The resulting super shifts could be analyzed by EMSA.

6. Analysis of IκBα

6.1 IκBα turnover

Whole cell extracts of COS-7 cells were normalized to 20 μg and electrophoresed on a 10% SDS polyacrylamide (19.2:0.8 crosslinking) denaturing gel. Protein was transblotted to nitrocellulose membrane (Hybond-C super; Amersham), which was blocked for 1hr with 2.5% skim milk dissolved in PBS. IκBα and actin were detected by incubating membranes for 1hr with rabbit polyclonal IκBα primary antibody (C-21; Santa Cruz, Inc) or mouse anti-actin monoclonal antibody (ICN) respectively. Membranes were washed repeatedly in PBS; 0.1% Tween and incubated in horse-radish peroxidase (HRP) conjugated anti-mouse or anti-rabbit secondary antibody (Kirkegaard & Perry Laboratories) for 1hr. Membranes were washed in PBS; 0.1% Tween and specificity was determined by chemiluminescence detection of the HRP-conjugated complexes (Dupont) according to the manufacturer's instructions.

6.2 Phosphorylation of IκBα

Whole cell extracts were prepared in the same fashion except that the non-specific phosphatase inhibitor okadaic acid (20 μM) was used to prevent dephosphorylation of IκBα. As well, to avoid inducible degradation of IκBα, 30 μM of MG132 was pre-incubated for 30 minutes prior to stimulation with TNF-α, or PMA. Protein extracts were normalized to 20 μg and resolved using 15% SDS polyacrylamide denaturing electrophoresis. Proteins were subsequently transferred to nitrocellulose membrane and detected using the mouse monoclonal IκBα (MAD10B) antibody as previously described. Phosphorylated IκBα is detected as a slower migrating species in comparison to unphosphorylated IκBα.

7. Transient Transfection

7.1 Calcium Phosphate Transfection

293 cells were transiently transfected using the calcium phosphate procedure (204). The precipitated DNAs (10 µg), representing either HIV Enhancer CAT or mutated HIV Enhancer CAT were resuspended in 450 µl of sterile water and 50 µl of 2.5 M $CaCl_2$. The DNA/$CaCl_2$ mixture was slowly added to a solution of 2x HEPES buffered saline. This solution was incubated at RT for 20 minutes to allow precipitated $CaCl_2$/DNA complexes to form. Precipitates were inoculated onto culture dishes and incubated for 4-16 hr under standard growth conditions. Cells were washed twice with 1x PBS and refed complete medium. At 48 hr post-transfection, cells were treated with the various drugs and harvested.

7.2 Lipofectamine Transfection

NIH 3T3 cells were plated to a density of apporoximately 80% confluence. Plasmid DNA was precipitated and resuspended in 1.6 ml of serum free Dulbecco's Modified Eagle's Media (DMEM). This mixture was incubated with 30 µl Lipofectamine liposomes (GIBCO) at room temperature for 30 min. After incubation, the resulting lipofectamine/DNA complexes were added to cells and the transfection allowed to proceed for 5-8 hours, afterwhich cells were washed with DMEM and incubated for 48 hours before beginning experiments.

8. Chloramphenicol acetyltransferase (CAT) assay

At 48 hrs post transfection , cells were treated with various drugs and inducers and the cells collected as previously described. Cells were resuspended in 0.25 M Tris-HCl pH 7.5 and lysed by three successive cycles of freeze thaw. Cells were centrifuged at 10, 000 g for 10 min and the supernatant collected. Soluble protein was quantified by Bradford

assay (Bio-Rad). Equal amounts of protein (20-100 μg) were assayed for CAT activity in the presence of 25nCi [^{14}C] chloramphenicol and 0.7 mg/ml acetyl CoA for 4-6 hrs. depending on the level of inducible CAT expression. The chloramphenicol and acetylated chloramphenicol were purified by extraction with 1 ml of ethyl acetate and centrifuged at 10.000 g for 10 min. The organic phase was removed and Speedvac evaporated. Samples were resuspended in 30 μls of ethyl acetate and blotted onto a silca-based thin layer chromatography sheet. Acetylated chloramphenicol was resolved using a 19:1 chloroform/methanol solvent and running the solvent front 1mm from the top of the sheet. CAT activity was measured by liquid scintillation and inducibility of CAT expression was calculated as the relative fold-induction over that of the untreated control.

9. PKC Assay

NIH 3T3 cells were grown to a confluency of 80% and incubated at 37°C for the indicated times in the presence of PMA with or without taxol. Whole cell extracts were prepared as described above except that 20 μM sodium vanadate was used to prevent dephosphorylation of the PKC substrate KRTLRR (Sigma), an epithelial growth factor (EGF)-derived peptide (56). Using 50 μg of whole cell extract, reactions were incubated with 500 mM MOPS, pH 7, 100 mM $MgCl_2$, 2.5 mg/ml BSA, 0.2 μCi [γ^{32}P] ATP, and 1 mM PKC substrate. A background control was setup containing only the lysis buffer as a substitute for whole-cell extract and the resulting value subtracted from each of the experimental measurements. Reactions were incubated for 1 hour at 37 °C. Reactions were stopped with 50% trichloroacetic acid (TCA) and incubated on ice for 10 min. Cells were centrifuged at 14, 000 x g for 10 min and the resulting supernatants were applied to phospho-cellulose paper followed by three washes with 0.4% o-phosphoric acid. PKC activity was quantitated as the relative fold increase in the level of [γ^{32}P] ATP incorporated into the PKC substrate.

10. Indirect Immunoflourescence of Cytoplasmic Microtubules

Immunofluorescence of COS-7 cells was performed as follows. Cells were seeded at a density of 10^5 cells in six well plates each containing glass coverslips. Cells were allowed to adhere to the surface of plate, approximately 8 hrs. After treatment, coverslips were removed and washed twice with 1X PBS. Cells were subsequently fixed with 4% paraformaldehyde for 15 minutes followed by washing with PBS. Cells were permeabilized with 0.3% Triton X-100 in PBS for 5 min. and washed three times with PBS. Cells were blocked for 30 min. in 5% BSA and incubated with mouse monoclonal tubulin antibody (Sigma) (dilution 1:250) for 2 hrs. After incubation with the primary antibody, cells were washed numerous times with 1X PBS and incubated for 1 hr with mouse-FITC (1:250 dilution) (Jackson Laboratories) conjugated antibody followed by three washes with 1X PBS. Microscopy was performed using a Leitz fluourescence microscope (Aristoplan).

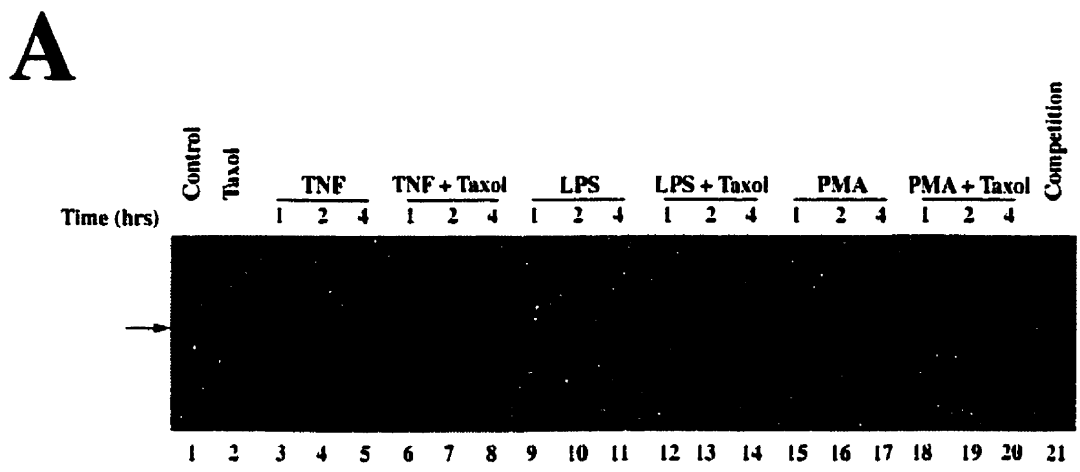
RESULTS

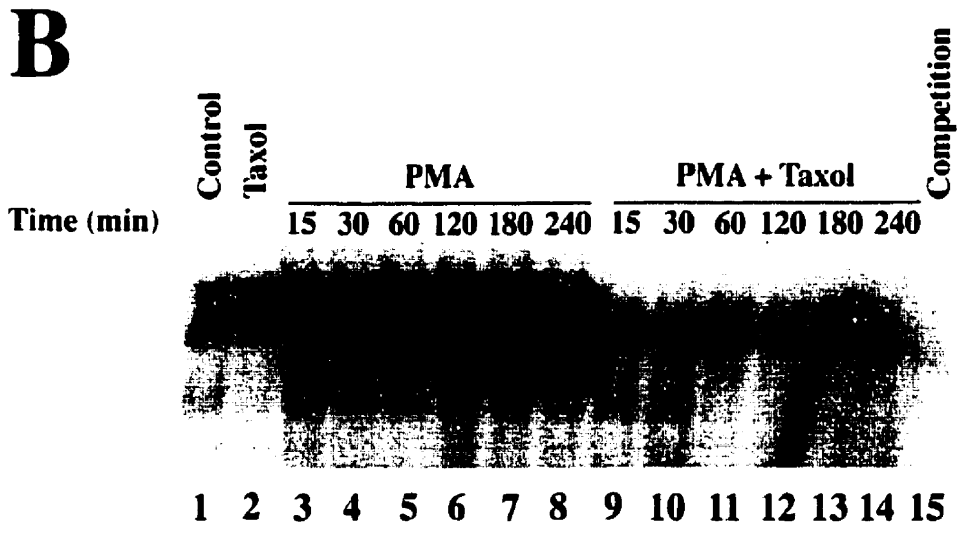
1. Taxol Specifically Suppresses PMA-induced NF-κB Activation

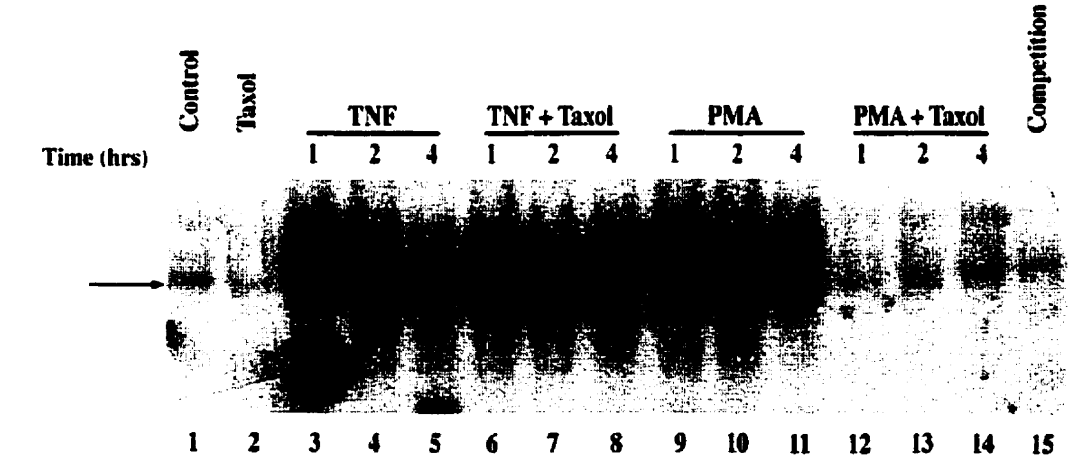
Previous reports have established taxol as a potent stabilizer of microtubule integrity (60). In order to better understand the effects of taxol as a microtubule stabilizing agent, experiments were undertaken to determine whether taxol could affect transcriptional activation of NF-κB in adherent cell populations. Nuclear extracts were examined for NF-κB-binding activity by mobility shift assay, using the PRD II domain of the IFN-β promoter (107). NF-κB DNA-binding activity was examined in NIH 3T3 mouse fibroblasts as well as human 293 embryonic kidney cells. Initial observations in NIH 3T3 cells (Figure 10A, lane 2) suggested that pretreatment of taxol had the potential to suppress constitutive NF-κB DNA binding and also selectively inhibited PMA induced NF-κB activation (Figure 10A, lanes 15-20). In contrast, taxol had no effect on TNF-α stimulation of NF-κB DNA binding (Figure 10A, lanes 3-8), nor was taxol capable of overriding the LPS hyporesponsiveness in 3T3 cells, a characteristic common to mouse fibroblasts (104, 105, 106). To determine whether taxol could block PMA at earlier stages of NF-κB activation by PMA (Figure 10B), taxol and PMA were added simultaneously and the kinetics measured at earlier time points. Interestingly, taxol effectively blocked PMA stimulation as early as 15 minutes and maintained its suppressive effects as late as four hours (Figure 10B, lanes 9-14). Discrepancies between panel A and B of Figure 10 represent two different batches of 3T3 cells and may explain the differences in kinetic behaviour of NF-κB binding in the presence of PMA. Also, comparison of Figure 10A and 10B suggested that the pretreatment or simultaneous treatment of taxol had similar inhibitory effects on PMA induction.

In order to determine whether taxol inhibition of PMA induced NF-κB DNA binding was a generalized response in adherent populations or specific to mouse fibroblasts, the

Figure 10. The effect of taxol on the activation of NF-κB by TNF-α, LPS, and PMA. NIH 3T3 cells were treated with either 10 ng/ml TNF-α, 10 ng/ml LPS, or 100 ng/ml PMA for the indicated times in the presence or absence of 5 μM taxol. NF-κB DNA binding was measured using 5 μg of nuclear protein extract incubated with a ^{32}P labeled DNA probe as described in Methods and Materials. NF-κB specific complexes are indicated by the arrow. (A) Effect of taxol on the DNA binding potential of NF-κB in the presence of various inducers. Cells were preincubated with 5 μM taxol for 30 minutes (lanes 2, 6-8, 12-14, and 18-20). The specificity of the band corresponding to NF-κB binding was measured using 125M excess of unlabeled cold DNA probe to compete away the specific complexes. (B) Similar experiment using a shorter time course for PMA inducible NF-κB DNA binding except that taxol treated cells (lanes 2, 9-14) were not pretreated. (C) Using a similar approach as (A), 293 cells were treated with either TNF-α (10 ng/ml) or PMA (100 ng/ml) and either untreated (lanes 3-5 and lanes 9-11 respectively) or pretreated with 5 μM taxol (lanes 6-8 and lanes 12-14 respectively). Cold competition is shown in lane 15.


A
Control
Taxol
TNF
TNF + Taxol
LPS
LPS + Taxol
PMA
PMA + Taxol
Competition
Time (hrs)
1 2 4 1 2 4 1 2 4 1 2 4 1 2 4 1 2 4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21


B
Control
Taxol
PMA
PMA + Taxol
Competition
Time (min)
15 30 60 120 180 240 15 30 60 120 180 240
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


C
Control
Taxol
TNF
TNF + Taxol
PMA
PMA + Taxol
Competition
Time (hrs)
1 2 4 1 2 4 1 2 4 1 2 4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

inhibitory role of taxol in DNA-binding assays in 293 human embryonic kidney cells (Figure 10C) was examined. Comparable to NIH 3T3 cells (Figure 10C; lane 9-14), PMA stimulation of NF-κB DNA binding was inhibited by taxol, yet demonstrated no suppressive effects on TNF-α stimulation (Figure 10C; lanes 3-8).

2. Taxol activates NF-κB DNA binding at higher concentrations

It has been previously demonstrated that higher concentrations of taxol are capable of inducing NF-κB activation (99,37). Figure 11 (lane 2) outlines the effect of 10 μM taxol on NF-κB DNA binding in NIH 3T3 cells. As expected, exposure of NIH 3T3 cells with 10 μM taxol for 4 hours resulted in a level of inducible NF-κB DNA binding higher than that obtained for PMA at 4 hours (lane 5), suggesting the concentration dependent effects of taxol are consistent with previous observations (168).

3. Characterization of NF-κB subunits

To further characterize the nature of the NF-κB-DNA complexes generated after PMA induction, specific antibodies directed against either RelA (p65), p50/p105, or c-Rel were used in a supershift assay to determine the subunit composition of the NF-κB complexes (Figure 12). Supershifting was accomplished by pretreating nuclear extracts with the various antibodies prior to addition of the radioactive probe. The two major NF-κB complexes identified were RelA(p65) and c-Rel (Figure 12, lanes 4 and 5 respectively). The intensity of the NF-κB complex corresponding to RelA(p65) was 20-fold higher than the c-Rel shifted complex. The relative excess of p65 over c-Rel, suggests that PMA induction of NF-κB recruits predominantly p65 homodimers and to a lesser extent p65/c-

Figure 11. Taxol activates NF-κB DNA binding at higher concentration.

NIH 3T3 cells were incubated with 10 μM taxol for 4 hours (lane 2) and NF-κB DNA binding activity assessed. Untreated cells are shown in lane 1, while PMA induced cells (1-4 hours; lanes 3-5) are shown as a positive control for NF-κB binding. Cold competition (lane 6) displays specificity of NF-κB binding.

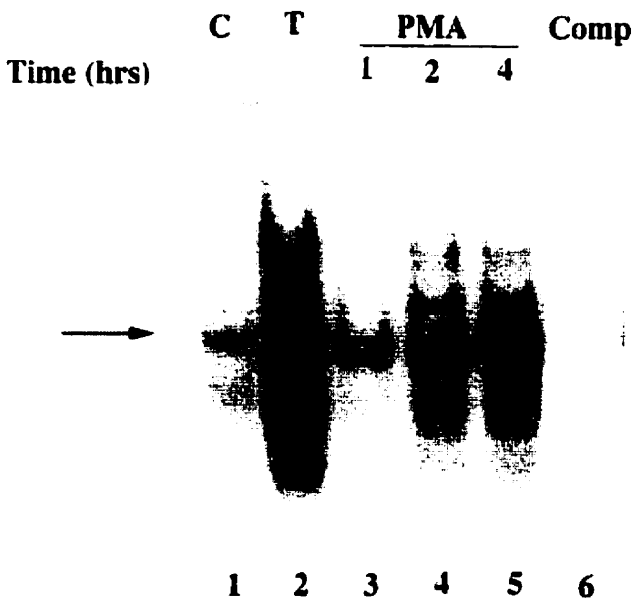
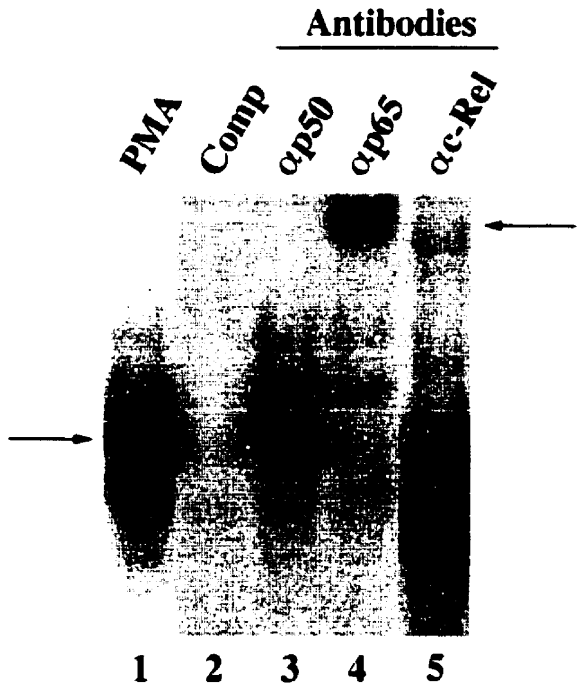

C
T
PMA
Comp
Time (hrs)
1
2
4
1
2
3
4
5
6

Figure 12. Subunit characterization of PMA induced NF-κB-DNA complexes. Nuclear extracts from NIH 3T3 induced for 4 hrs with PMA (lanes 1-5) were incubated with murine specific antibodies recognizing the NF-κB subunits p50 (lane 3), p65 (lane 4), and c-Rel (lane 5). The lower arrrow indicates NF-κB specific complexes and the upper arrow indicates the antibody shifted complexes.

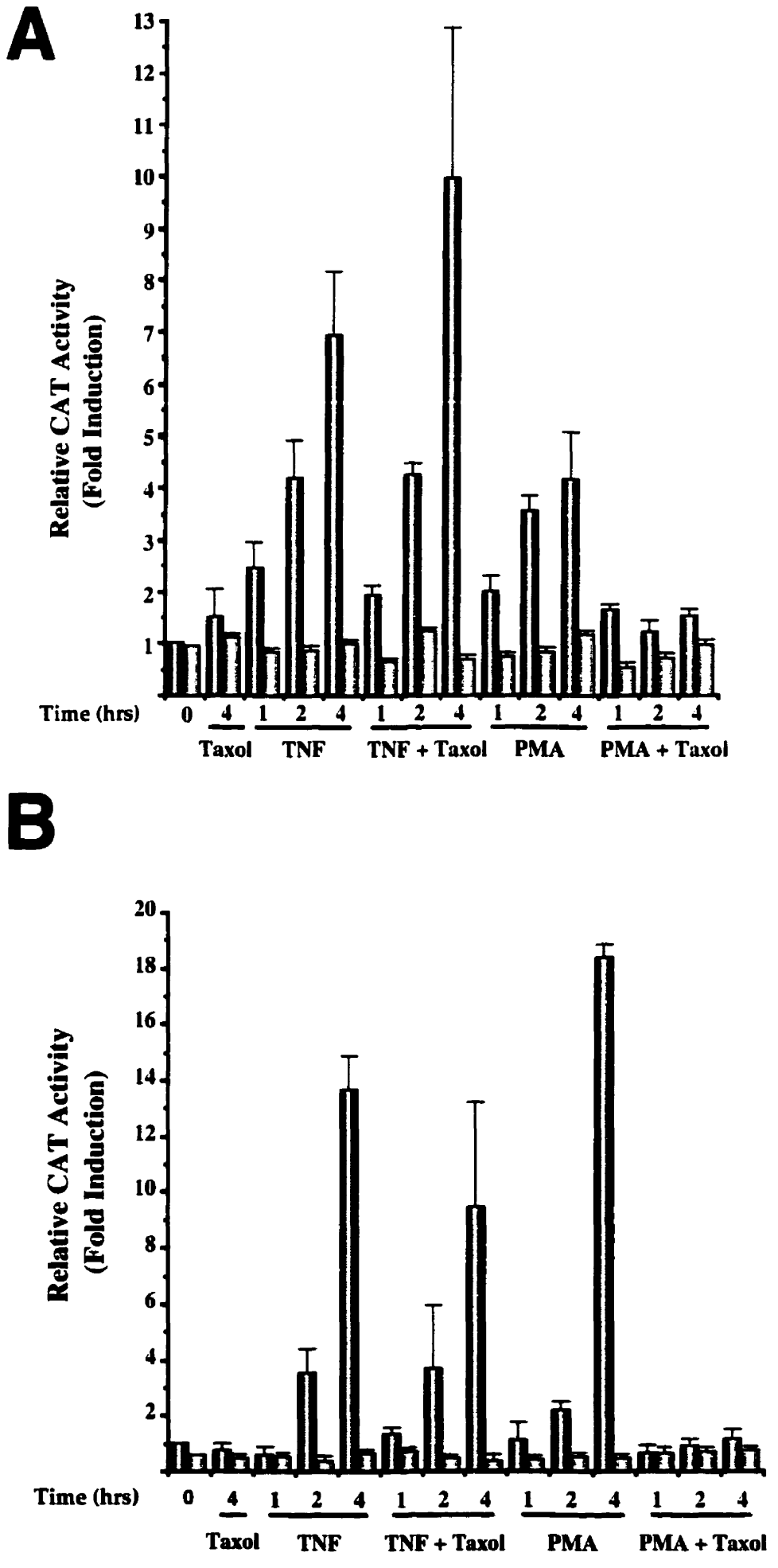

Antibodies
PMA
Comp
αp50
αp65
αc-Rel
1
2
3
4
5

Rel heterodimers. Surprisingly, no p50 subunits could be identified in the supershift assays.

4. Taxol also suppresses NF-κB-specific gene expression

The inhibitory effect of taxol on PMA and TNF-α induced NF-κB dependent gene activity was analyzed using the HIV Enhancer CAT plasmid. Both 3T3 and 293 cells were transfected with HIV Enhancer CAT plasmid as well as the mutated HIV Enhancer CAT plasmid to determine the specificity of NF-κB transactivation (Figure 13). In 293 cells transiently transfected with HIV Enhancer CAT, TNF-α stimulation increased reporter gene expression by 7-fold (Figure 13A, lanes 3-5), while TNF-α in the presence of taxol increased gene expression by 10-fold. In the case of PMA, the inducibility of CAT expression was much lower (Figure 13A, lanes 9-11) only reaching a 4-fold level of induction. Consistent with the EMSA data, the level of CAT expression was repressed in PMA stimulated cells pretreated with taxol (Figure 13A, lanes 12-14) to levels comparable with control CAT expression. Similar results were also obtained for NIH 3T3 cells except that in the TNF-α treated lanes (Figure 13B, lanes 3-5), the fold inducibility of CAT activity reached a higher value (14-fold) while the TNF-α + Taxol (Figure 12B, lanes 6-8) was similar to that obtained for 293 cells. However, PMA stimulated 3T3 cells showed a significant difference in the level of gene expression for cells treated with either PMA (Figure 13B, lanes 9-11) or PMA + taxol (lanes 12-14). While cells treated with PMA alone displayed a 20-fold increase in CAT expression, the presence of taxol diminished CAT expression to levels below those for the control lane (Figure 13B, lane 1). One other notable difference concerned the level of CAT expression in cells treated only with taxol. In 293 cells (Figure 13A, lane 2), taxol increased the level of CAT expression about 1.5 fold, on the other hand, taxol decreased the level of basal CAT expression. Finally, to determine that NF-κB was indeed required for CAT

Figure 13. Effect of taxol on HIV Enhancer-mediated gene expression. NIH 3T3 and 293 cells were transfected with 10 μg of HIV Enhancer CAT plasmid, which contains three copies of the NF-κB consensus DNA binding site or HIV Enhancer Mutant CAT plasmid, which has the NF-κB sites mutated. Transfected cells were pretreated with or without 5 μM taxol 48 hours post-transfection in the presence of either TNF-α or PMA. The level of HIV Enhancer-driven transcription was determined by CAT assay on the total cell extract. Results shown represent the average of three experiments. (A) Relative CAT activity in 293 cells stimulated with 100 ng/ml PMA (or 10 ng/ml TNF-α in the presence or absence of 5 μM taxol. (B) Relative CAT activity in NIH 3T3 under similar conditions as above. ☐ HIV Enhancer CAT. ▨ HIV Enhancer Mutant CAT.


A
Relative CAT Activity
(Fold Induction)
13
12
11
10
9
8
7
6
5
4
3
2
1
Time (hrs)
0 4 1 2 4 1 2 4 1 2 4 1 2 4
Taxol
TNF
TNF + Taxol
PMA
PMA + Taxol
B
Relative CAT Activity
(Fold Induction)
20
18
16
14
12
10
8
6
4
2
Time (hrs)
0 4 1 2 4 1 2 4 1 2 4 1 2 4
Taxol
TNF
TNF + Taxol
PMA
PMA + Taxol

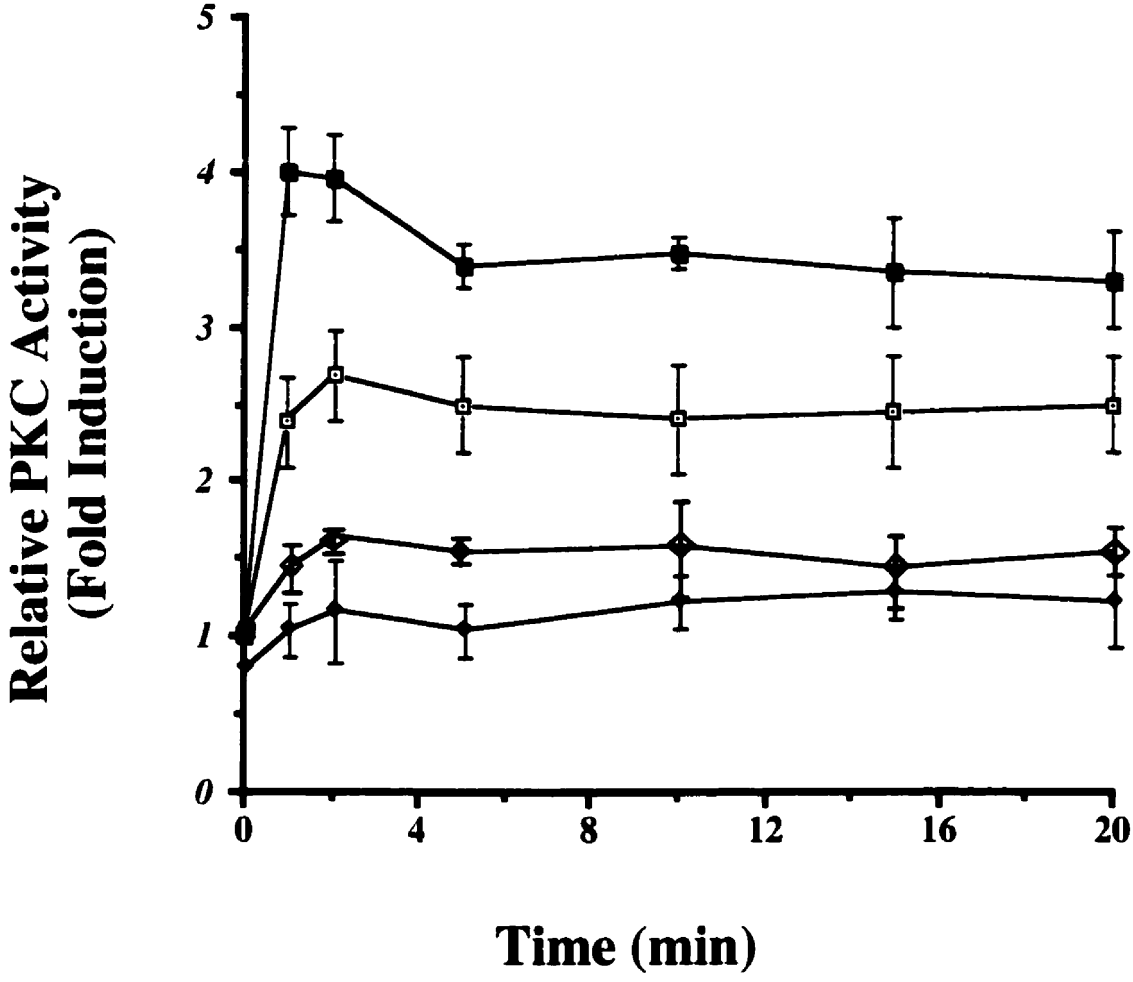
expression in the above CAT assays, a mutated HIV Enhancer CAT plasmid which contains mutations in the two NF-κB consensus sites was transfected into cells and the level of CAT expression measured under similar conditions (Figure 13A and 13B, striped boxes). As shown, the level of CAT inducibility in all lanes did not exceed that of untreated controls, demonstrating that an intact NF-κB binding site is required for reporter gene expression.

5. Taxol modulates the activity of PKC

The key upstream element in signaling by PMA is protein kinase C (PKC) (56). To determine whether activated PKC is a target for taxol, an *in vitro* assay was designed to measure the activation potential of PKC. Whole cell extracts of PMA treated NIH 3T3 cells were incubated with a peptide fragment corresponding to the EGF receptor, a substrate for conventional PKCs (Figure 14). In order to limit the level of background labeling of endogenous substrates present in the whole cell extracts, reactions were precipitated with TCA. Due to the relatively small size of the EGF peptide, it remains soluble in TCA and can be effectively purified. The peptide is subsequently immobilized on phosphocellulose paper and the level of phosphorylation determined. In the case of PMA treated cells, PKC activity reached a 4-fold maximum induction between 1-4 minutes (Figure 14; closed box). In cells pre-treated with taxol, the maximum induction was approximately 1.5 fold lower than that of cells treated without taxol (Figure 14; open box). Thus, the inhibitory effects of taxol may block NF-κB activation by suppressing PMA-inducibility of PKC. Since TNF-α is not a conventional activator of PKC, the basal level of PKC activity was relatively unaffected in the presence of TNF-α (Figure 14; open diamond) regardless of whether taxol was present (Figure 14; closed box) or not

.

Figure 14. Effect of taxol pretreatment on the relative activity of PKC in the presence of PMA. Whole cell lysates of NIH 3T3 cells (50 µg) were treated with 100 ng/ml PMA for the times indicated in the presence or absence of 5 µM taxol. PKC activity was measured as the ability of PKC to incorporate ^{32}P-ATP into the peptide substrate KRTLRR corresponding to the EGF receptor as outlined in the Material and Methods. Results represent the average of four experiments. ◆ PMA, ■ PMA + Taxol., ⊡ TNF, ◊ TNF + Taxol


Relative PKC Activity
(Fold Induction)
5
4
3
2
1
0
0
4
8
12
16
20
Time (min)

6. PMA induces changes in microtubule architecture

Many studies have shown that PMA exerts changes in the microtubule cytoskeleton through its activation of PKC (75, 81, 82, 83, 86, 99). Since it is apparent that taxol suppresses PKC activity in the presence of PMA, we next sought to determine whether COS-7 cells would undergo changes in microtubule architecture when exposed to PMA and to what extent taxol could interfere with these inducible structural changes (Figure 15). Indirect immunofluorescence microscopy demonstrated that cells treated with taxol do not cause any major structural changes in the tubulin network as compared to untreated cells (Figure 15B and 15A respectively). As a comparison, nocodazole, a reversible inhibitor of tubulin polymerization (111) was used to demonstrate breakdown of the microtubule network (Figure 15C). As expected, taxol was capable of preventing tubulin breakdown in nocodazole treated cells (Figure 15D). PMA treated cells (Figure 15E) induced dramatic changes in microtubule structure that were inhibited by taxol addition. Neither TNF-α nor LPS were capable of inducing changes in cytoarchitecture (data not shown), suggesting PMA is a unique microtubule signaling drug.

Two major morphologies were present in PMA treated cells. First, a number of cells demonstrated a homogeneous breakdown of the tubulin matrix similar to nocodazole treated cells (Figure 15C) suggesting that PMA has the potential to cause transient depolymerization of microtubules. Second, cells displaying an intact microtubule network exhibited dramatic changes in cell shape, denoted by extended membrane projections not present in control cells. Taxol pretreatment of these cells (Figure 15E) demonstrated a strikingly different morphological outcome. Taxol treated cells exhibit the typical control cell morphology (Figure 15A). While the membrane projecting morphology was seen in the PMA/taxol treated cells, the projections were fewer and less

Figure 15. Indirect immunofluorescence measuring the effect of taxol, PMA, and nocodazole on α-tubulin organization. COS-7 cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. α-tubulin was detected using a FITC-conjugated mouse monoclonal antibody (green). Cells were treated with either 20 μM nocodazole (panels C and D) or 100 ng/ml PMA (panels E and F) for 1 hr in the presence (panels B. D. and F) or absence (panels C and E) of 5 μM taxol. Untreated cells are shown in panel A.

dramatic. At the same time, no cells could be identified which displayed depolymerized microtubules. These results are consistent with the notion that taxol can suppress PMA-induced NF-κB transactivation by repressing PKC activation and blocking subsequent changes in microtubule structure.

7. Taxol suppresses both IκBα phosphorylation and turnover

It has been well characterized that inducible degradation of IκBα precedes NF-κB translocation to the nucleus and activation of NF-κB specific genes (16). Therefore to determine if taxol indeed suppressed PMA inducible NF-κB DNA binding, we next established whether taxol could alter the inducible turnover and phosphorylation of IκBα.

To measure turnover rates of IκBα, COS-7 cells were pretreated with cycloheximide in order to block protein synthesis and thus the re-synthesis of IκBα. After induction of cells, IκBα and actin were detected by immunoblot analysis. As previously shown, PMA effectively induced degradation of IκBα by 2 hours (Figure 16, lanes 3-6) while taxol treated cells showed no inducible degradation in the presence of PMA (Figure 16, lanes 7-10). This experiment demonstrated that the ability of taxol to block PMA-induced IκBα degradation correlated with taxol inhibition of PMA-induced NF-κB DNA binding.

Phosphorylation of IκBα on serine 32 and 36 is the critical event controlling downstream proteolytic degradation of IκBα and NF-κB activation (108, 109, 110). To maintain the phosphorylated state of IκBα, the proteasome inhibitor MG132 was used to block the degradation of IκBα, thus allowing the accumulation and detection of phosphorylated IκBα (Figure 17). After drug treatment of COS-7 cells, phosphorylated IκBα was separated from unphosphorylated IκBα by 15% SDS-PAGE and the resulting proteins

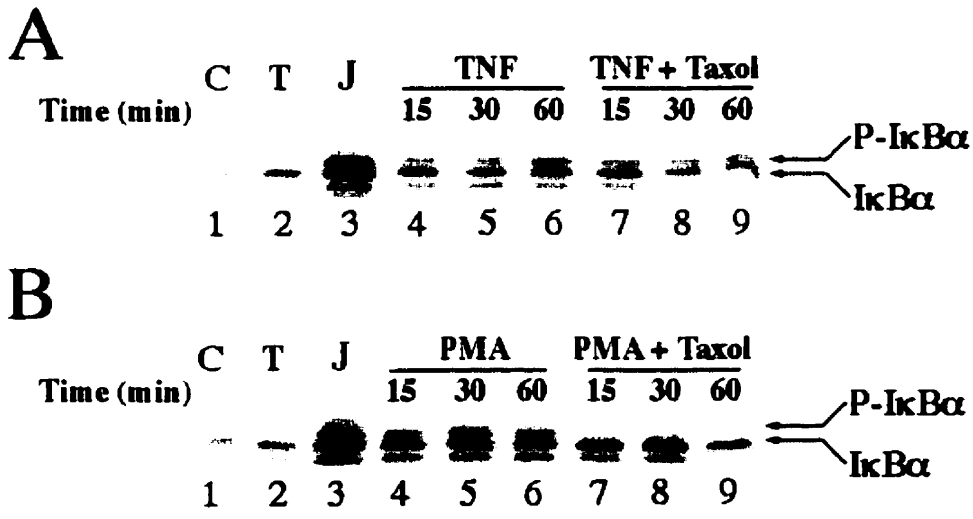

A
C T J TNF TNF + Taxol
Time (min) 15 30 60 15 30 60
P-IκBα
IκBα
1 2 3 4 5 6 7 8 9
B
C T J PMA PMA + Taxol
Time (min) 15 30 60 15 30 60
P-IκBα
IκBα
1 2 3 4 5 6 7 8 9

Figure 17. Immunoblot analysis of IκBα phosphorylation. COS-7 cells were treated with TNF-α or PMA (Fig. 4A and B respectively) in the presence or absence of 5 μM taxol (30 min. pretreatment). Whole cell extracts were normalized to 20 μg and separated by SDS-PAGE. IκBα was detected using a mouse monoclonal IκBα antibody (MAD10B). Jurkatt T-cells stimulated with 10 ng/ml TNF-α for 1 hr were used as a positive control (lane 3. Fig. 4A and B). (A) Cells were treated with 10 ng/ml TNF-α in the absence (lanes 4-6) or presence of 5 μM taxol (lanes 7-9). (B) Cells were treated with 100 ng/ml PMA in the absence (lanes 4-6) or presence of 5 μM taxol (lanes 7-9).

transferred to nitrocellulose. After immunoblotting with IκBα specific antibodies, the phosphorylated form of IκBα was identified as a slower migrating species just above unphosphorylated IκBα. As early as 15 minutes, the phosphorylated form of IκBα was detected in both TNF-α and PMA treated cells (Figure 17A, lanes 4-6 and Figure 17B, lanes 4-6 respectively). The pretreatment of taxol selectively inhibited the accumulation of phosphorylated IκBα in PMA treated cells (Figure 17A, lanes 7-9) while cells treated with TNF-α were unaffected by taxol (Figure 17B, lanes 7-9). In light of these results, taxol appears to be capable of blocking PMA induced phosphorylation of IκBα but not TNF-α stimulated phosphorylation.

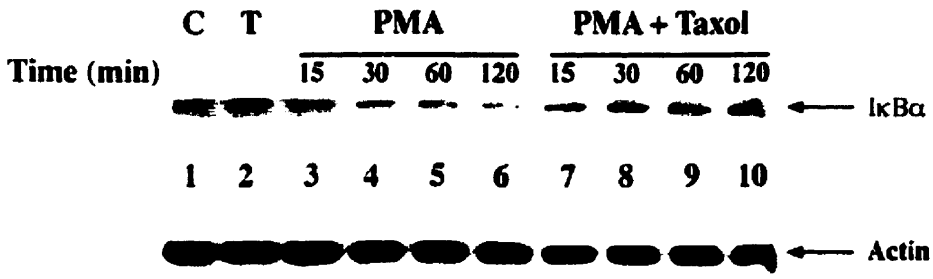

C
T
PMA
PMA + Taxol
Time (min)
15
30
60
120
15
30
60
120
IκBα
1
2
3
4
5
6
7
8
9
10
Actin

Figure 16. Immunoblot analysis of IκBα degradation in the presence or absence of taxol. COS-7 cells were cultured in the presence of cylcoheximide and were either untreated or pretreated 30 min. with taxol prior to stimulation with PM A. Protein extracts (30 μg) were resolved by SDS-PAGE, transblotted to nitrocellulose, and proteins were identified using specific antibodies. Immunoblot of IκBα degradation in COS-7 cells is shown. Cells were pretreated for 30 minutes with 5 μM taxol (lanes 2, 7-10), in the presence or absence of 100 ng/ml PMA for the indicated times. The respective levels of IκBα and actin are shown (top and bottom, respectively).

DISCUSSION

1. Summary

Previous studies have demonstrated the inhibition of NF-κB by other known terpenes such as circumin (162) and helenalin (8), suggesting the role for microtubules in NF-κB activation. The objective of these studies was to determine whether taxol could specifically interrupt NF-κB signaling in pathways known to involve microtubule reorganization. To further characterize the PMA pathway in NIH 3T3 cells, *in vitro* protein kinase C assays were used to determine whether taxol was effectively blocking NF-κB activity through this upstream kinase. Three major classes of NF-κB inducers were used, namely, LPS, TNF-α, and PMA, the latter being important in mediating the regulation of cytoskeletal architecture (83, 86). 1) NIH 3T3 and 293 cell models demonstrated that taxol could effectively inhibit NF-κB DNA binding activity in PMA induced cells but not in TNF-α and LPS stimulated cells; 2) Loss of NF-κB binding in taxol treated cells stimulated with PMA correlated with CAT reporter assays measuring NF-κB gene activation. 293 cells showed a somewhat marked decrease in PMA stimulated CAT expression while NIH 3T3 cells displayed a significant suppression of PMA inducible CAT activity in the presence of taxol; 3) IκBα phosphorylation was inhibited during PMA stimulation but not after TNF–α induction; 4) Turnorver of PMA treated COS-7 was also inhibited by the presence of taxol. 5) A 30% decrease in the level of PKC activity was measured in PMA stimulated cells pretreated with taxol.

Since taxol suppresses PMA stimulation early in the pathway, we wanted to determine whether down regulation of PKC activity may affect microtubule reconstitution in PMA treated COS-7 cells. As expected, COS-7 cells treated with PMA demonstrated a

substantial depolymerization of cytoplasmic microtubules along with dramatic changes in membrane shape. These findings were similar in nature to nocodazole treated cells, a known depolymerizing agent of microtubules, which caused the breakdown of mictotubules, giving the cells a more rounded appearance. However, in the presence of taxol, both nocodazole and PMA-induced MT depolymerization were prevented, and maintained a cytoarchitecture reminiscent of intact mirotubules.

The stabilization of microtubules by taxol was sufficient to block PMA-mediated rearrangement of the microtubule network and in turn suppress the activation potential of PKC. The capacity of taxol to modulate upstream events in the PMA pathway demonstrates the ability of taxol to block downstream transcriptional activation of NF-κB, suggesting taxol may also inhibit other transcription factors which rely on changes in the localized microtubule environment for successful activation. It has been shown previously that NF-κB/IκBα co-localized to microtubules through an interaction between the signal response domain of IκBα and the dynein light chain subunit of the dynein microtubule motor complex (34). Since latent NF-κB complexes are sequestered in the cytoplasm through their interaction with IκBα and microtubules, microtubule integrity may play an important role in regulating NF-κB activity by modulating its interaction with the microtubule network. This suggests that taxol-stabilized microtubules and NF-κB complexes maybe sufficient to prevent IκBα degradation and maintain NF-κB in the latent complex.

Based on these studies, it appears that taxol is capable of stabilizing microtubules from deassembly or rearrangement. In the case of PMA stimulation, taxol stabilization of microtubules appears to suppress the activation of PKC, an important upstream mediator involved in NF-κB activation. Thus, PMA stimulation of NF-κB activation is a microtubule dependent pathway that is sensitive to the presence of taxol. On the other

hand, TNF-α appears to demonstrate a microtubule-independent pathway which is not affected by taxol.

2. Microtubule Integrity and NF-κB Activation

In cases where microtubule stability is lost, such as pretreatment with nocodazole or colchicine, NF-κB is activated (147). This would suggest that loss of interaction with the microtubule network through dynein light chain may be sufficient to activate inducible degradation of IκBα. However, the notion that taxol regulates NF-κB activation at the level of the microtubule may only account for part of this observation. Taxol is also known to alter the balance of microtubule associated proteins (23). Taxol-treated cells develop abnormal bundling of MTs and several lines of evidence suggest that MAPs are responsible for influencing the spacings of microtubule (205); taxol may alter the MAP composition of these bundles. In cultured sympathetic neurons, treatment with taxol can diminish the association of a number of 60-76 kDa species of MAPs, indicating that taxol may diminish populations of MAPs important for cytoskeletal and signal transduction regulation (89).

Recently, a phorbol ester resistant U937 variant was shown to have diminished levels of microtubule-associated $PKC\beta_2$ due to the loss of microtubule-associated PKC binding proteins (88). Interestingly, depolymerization of microtubules with nocodazole was capable of reconstituting the normal phenotype. These findings suggest a role for $PKC\beta_2$ association with microtubules as a requirement for normal microtubule reorganization. Given this observation, it is possible to postulate that taxol could also have a similar effect on MT-associated PKC binding proteins in a fashion similar to that described above. In this case taxol may deplete the available pool of microtubule associated PKC thereby affecting downstream signals including IκBα phosphorylation. One candidate is

the PKC binding protein RACK1 (receptors for activated C-kinase 1) which is involved in anchoring activated PKCβ (or other PKCs) to the cytoskeleton (186) and is required for its activation. If indeed taxol is capable of preventing RACK1 association with the microtubule, this may represent a possible mechanism by which taxol suppresses activation of PMA stimulated PKC.

3. PKC is a major component of NF-κB signaling and cytoskeletal dynamics

PKCs represent a family consisting of at least 11 characterized members (68). PKCs can be subdivided into conventional (cPKC), novel (nPKC), and atypical (aPKC), all of which are activated by phorbol ester (155) except for PKCζ, which appears to require a conventional PKC, most likely PKCα, for its activation (39,60).

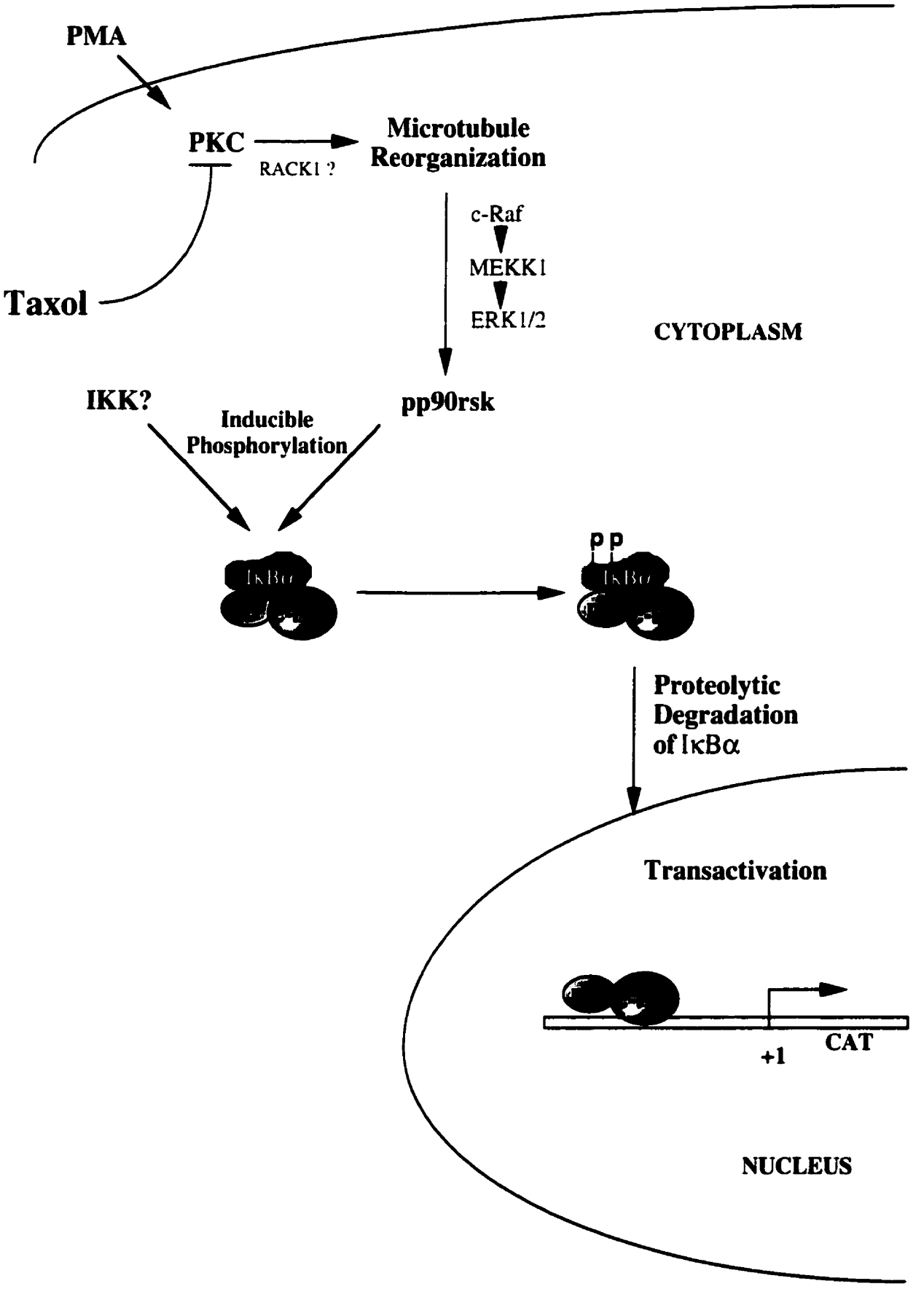
At present, numerous observations have established the requirement for PKC in cytoskeletal regulated differentiation and reorganization (7,40,46,84,107,123,126,174) particularly the actin and microtubule cytoskeleton. Treatment of metaphase II-arrested hamster eggs with PKC activators has been shown to promote resumption of the cell cycle (53). As well, stimulation of PKC in hamster eggs has also been demonstrated to induce polar body formation, increased actin polymerization, and breakdown of spindle microtubules (115). Treatment of mouse eggs with PKC activators does not result in spindle body formation or actin polymerization but does display microtubule disassembly. These findings demonstrate the importance of PKC as a mitotic re-activator and modulator of microtubule structure and is consistent with the results displaying PMA-induced microtubule reorganization of COS-7 cells (Figure 14).

Since an overall reduction in PKC activity was observed in taxol treated cells (see Figure 13), this may represent a specific decrease in the activity of taxol-sensitive PKCs and not a complete attenuation of all PKC family members, of which, PKC-α and PKC-ζ maybe taxol regulated subunits. Consistent with this idea is the observation by Bouron (1997) which suggests colchicine-mediated depolymerization of microtubules is sufficient to increase PKC activity. While PKC is able to induce dramatic changes in cytoskeletal assembly, an apparent feedback mechanism appears to exist whereby depolymerization of microtubules activates PKC, suggesting PKC activity is in part activated by external (ie PMA) and internal (ie cytoskeletal environment) stimuli. Thus the presence of taxol would not only block microtubule depolymerization of nocodazole and PMA-treated cells but may induce a cytoskeletally-inherent negative feedback effect on PKC activity (Figure 18).

4. PKC and Apoptosis

Interestingly, PMA stimulation of PKCs has known anti-apoptotic effects (170). As well, the phosphoinositol 3-kinase an upstream activator of PKCε (52) is also a pathway involved in the anti-apoptotic response. These findings provide evidence of a PKC-dependent mechanism for apoptotic rescue. Furthermore, NF-κB has long been identified as an inhibitor of apoptosis (17,103). A PKC-mediated response may involve activation of NF-κB to synergize a possible anti-apoptotic effect. In contrast, taxol is a potent stimulator of apoptosis (24,63,64). Given the antagonistic roles of taxol and PKC/NF-κB in apoptotic onset, it is not surprising that the strong apoptotic effects of taxol may be the result of the anti-mitotic effects of taxol in combination with its ability to suppress PKC and hence NF-κB activity. That is, taxol may not only induce apoptosis by itself but prevent the activation of anti-apoptotic mediators like NF-κB

Figure. 18. Schematic representation depicting how taxol suppresses PMA-induced NF-κB transactivation through its ability to block PKC activation and subsequent microtubule reorganization.


PMA
PKC
RACK1 ?
Microtubule Reorganization
c-Raf
MEKK1
ERK1/2
Taxol
CYTOPLASM
IKK?
Inducible Phosphorylation
pp90rsk
pp
IκBα
Proteolytic Degradation of IκBα
Transactivation
CAT
+1
NUCLEUS

The Taxol Paradox

Taxol has been shown to affect a number of upstream kinases, particularly PKC. In many cases, PMA induced expression of a number of genes including human CD32, CD16, CD35 (102,161), were specifically blocked by the addition of taxol. The paradox surrounding taxol is its concentration dependent effects. At low concentrations, taxol appears to elicit a cytotoxic effect but at higher concentrations has a positive regulatory effect especially in macrophage cells where taxol acts as an LPS-like activator in the priming of macrophages (81,168,188). Whether taxol is cell line specific or whether it conveys unique cellular effects at varying concentrations has not been established.

The results presented here contradict numerous findings establishing taxol as an activator of NF-κB, particularly in cancer tissue-derived cell lines and macrophage cell lines. A major difference between other studies and the present work revolves around the relative concentrations of taxol used. Taxol when used at a concentration of 5 μM is capable of suppressed IL-8 production (160) but at 30 μM taxol activated IL-8 expression through induction of NF-κB and AP-1 (99). Another study found that NF-κB could be activated in the presence of 97 μM taxol (37); similarly for taxol stimulation of macrophages, taxol concentrations in excess of 10 μM had stimulatory effects on ions and TNF-α secretion in macrophages (81,168). Our preliminary experiments have also been able to demonstrate that higher concentrations of taxol (~10 μM) had a positive regulatory effect on NF-κB (Figure 10). These findings suggest that taxol displays concentration dependent effects which appear to be divided among its microtubule stabilizing effect, which could suppress PMA stimulation of NF-κB , and its cytopathic effects, which represent a more generalized response.

The ability of taxol to induce tumor regression and apoptosis is strongly substantiated although its secondary effects on cellular processes have not been well investigated enough to generate a clear consensus of the physiology of this drug. Subsequent experiments should examine the differential effects of taxol to delineate the various cellular effects that taxol exhibits at varying concentrations

Further studies to determine which isoforms of PKC are directly affected by taxol are required; in addition, the role of other cytoskeletal-associated proteins such as RACK1 in taxol-mediated suppression of PKC are required. Finally, whether microtubule integrity is a necessary component of PKC activation remains to be determined. A number of subunits in the PMA pathway are microtubule bound complexes, such as ERK1 and 2, and it would be interesting to determine whether the inducible activation of these kinases is also affected by taxol. The results presented in this study outline an intriguing association between microtubule architecture and the transcriptional regulation of NF-κB.

Conclusions

The results of this study address a number of critical questions regarding the regulatory behavior of taxol. First, taxol displays a possible bipartite mechanism of cellular control which may or may not be a matter of concentration. Secondly, a number of questions still remain as to the functional significance of taxol as a mediator of apoptosis and an activator/repressor of signaling pathways especially PKC and NF-κB since taxol demonstrates a number of paradoxical activities in this regard. Furthermore, suppression of PKC by taxol may represent a single regulatory step in the down-regulation of NF-κB and other kinases such as ERK1/2, RSK, IKK, or the nuclear translocation machinery may also be taxol-sensitive targets as well. It should not be ruled out however, that the ability of taxol to display microtubule stabilizing effects may represent one of a multitude of cellular effects, suggesting novel regulatory pathways may be discovered which are regulated by taxol independent of the requirement for microtubules.

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