THE BIOCHEMICAL AND CELL CYCLE EFFECTS OF THE ANTITUMOUR DRUG FOSTRIECIN

by

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Abstract

Fostriecin is an antitumour drug in phase I clinical trials. It has been recently shown that it is a potent inhibitor of protein phosphatases 1 and 2A *in vitro*, a property not previously described for an antitumour drug. We have investigated its effects on protein phosphorylation in Baby Hamster Kidney cells. Fostriecin strongly stimulated the phosphorylation of a single protein, which we identified as the intermediate filament vimentin. Fostriecin also caused rounding of the cells and a reorganization of the vimentin filaments. These effects are similar to those of the known inhibitors of protein phosphatase 1 and 2A, okadaic acid and calyculin A, which are actually tumour promoters. Fostriecin induced hyperphosphorylation of vimentin mostly at two sites which were sensitive to staurosporine and could be phosphorylated by protein kinase C *in vitro*. Fostriecin-induced hyperphosphorylation of vimentin also occurred in cells that lack cdc2 activity. These results suggest that protein kinase C plays a role in the hyperphosphorylation of vimentin during exposure to fostriecin. Furthermore, they provide strong evidence that fostriecin inhibits protein phosphatases 1 and 2A *in vivo* and raise the possibility that it may have tumour promoting activity.

We further studied the effects of fostriecin on the cell cycle. When two human cancer cell lines, Hela and Jurkat, were treated with fostriecin, premature degradation of cyclin A and cyclin B was induced independently of mitosis. This degradation pathway required the activity of staurosporine-sensitive kinase(s) and ubiquitin-proteasome activity. These results suggest that protein phosphatase activity is required for the proper timing of cyclin

degradation. Upon fostriecin treatment and phosphatase inhibition, the degradation pathway is activated and cyclins are degraded. Since cyclins are essential for the progression of the cell cycle, we determined whether this premature cyclin degradation caused the cells to die. Instead we found that general cysteine neutral protease activity was required in part for the cytotoxicity of fostriecin and the degradation of other proteins besides cyclins is required for cell death.

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List of Abbreviations

ATP	adenosine triphosphate
BHK	baby hamster kidney
BSA	bovine serum albumin
CAK	cdk-activating kinase
CDI	cyclin-dependent kinase inhibitors
cdk	cyclin-dependent kinase
CLL	N-benzyloxycarbonyl-leu-leu-leucinal
DMEM	Dulbecco's minimal essential medium
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
FBS	fetal bovine serum
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP	horse radish peroxidase
IF	intermediate filament
LnLL	N-acetyl-leu-leu-norleucinal
MAP	mitogen activated protein
MPF	maturation-promoting factor
PAA	phosphoamino analysis
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate-buffered saline
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PSI	N-benzyloxycarbonyl-Ile-Glu(O-t-butyl)-Ala-leucinal
PP1	protein phosphatase 1
PP2	protein phosphatase 2
Rb	retinoblastoma protein
RPA	replication protein A
SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
TCA	trichloroacetic acid
TLC	thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane

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Chapter 1

Introduction

1.1 Cell Cycle

The cell cycle can be defined as the ordered set of processes by which one cell grows and divides into two daughter cells. Cell growth and division are fundamental events in biology. In recent years, scientific research in the cell cycle field has expanded dramatically. The implications of cell cycle research range from answering basic scientific questions to finding cures for cancer. Some aspects of the cell cycle have been elucidated, but many more questions remain unanswered.

Generally, the cell cycle can be divided into two fundamental parts: mitosis, the process of cellular division which is highlighted by the separation of duplicated chromosomes into two genetically identical nuclei, and interphase, the period of time between divisions when the cell grows and replicates its DNA. Interphase can be further divided into three phases: G1, the initial growth phase; S, the DNA synthesis phase, when each chromosome is replicated; and G2, the second growth phase when the cell prepares for mitosis (Fig. 1). In a typical animal cell cycle, G1 phase lasts twelve hours, S phase six hours, G2 phase six hours and mitosis one half hour (Murray, 1994). Not surprisingly the cell cycle is a tightly regulated process. The timing of each cellular event must be appropriate for successful cellular reproduction. There exist many regulatory and feedback mechanisms to monitor the cell cycle and prevent genetic damage, abnormal growth and even cell death.

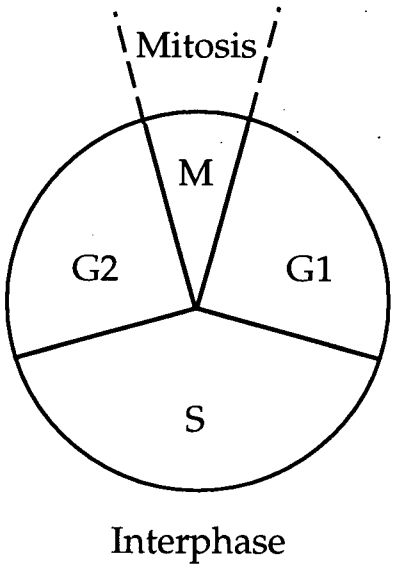


Figure 1. The eukaryotic cell cycle.

The eukaryotic cell cycle is divided into two major stages: mitosis and interphase. Interphase may be further divided into two growth phases, G1 and G2, and a DNA synthesis phase, S.

1.2 Cyclin-Dependent Kinases

Progression through the cell cycle is controlled by the cyclin-dependent kinases (cdks). The cdks are the central and rate-limiting controls in the regulation of each phase of the cell cycle (Dorée and Galas, 1994; Dunphy, 1994; Solomon, 1993). The transitions between phases in the cell cycle are controlled by changes in activity of cdks that phosphorylate specific targets. The cdk catalytic components constitute a family and their activity depends on association with positive regulatory subunits, the cyclins (Draetta, 1993; King et al., 1994; Murray, 1994) and negative regulatory subunits, the cyclin-dependent kinase inhibitors (CDI) (Elledge and Harper, 1994; Peter and Herskowitz, 1994).

Several cdks and cyclins have been discovered in recent years (Table 1). The different cdks regulate different phases of the cell cycle. Specifically, cdc2 regulates the entry and exit from mitosis and is associated with the mitotic cyclins A and B. The other cdks, except for cdk3 and cdk7, regulate other phases of the cell cycle, particularly the transition between the G1 and S phases, and are associated with the G1/S cyclins A, D and E.

Table 1-Vertebrate cdks and cyclins[a]

cdk	Associated cyclins
cdc2	A, B
cdk2	A, D, E
cdk3	?
cdk4	D
cdk5	D
cdk6	D
cdk7	H

[a]Ref: (Dorée and Galas, 1994; Draetta, 1994; Nigg, 1993)

1.2.1 Activation of cdk complexes

For optimal activity, cdks associated with cyclins also need to be phosphorylated at certain residues. Cdc2-cyclin and cdk2-cyclin complexes are phosphorylated at Thr 161, a residue that is highly conserved in all members of the cdk family. The activity of the kinase increases approximately 100-fold when Thr 161 is phosphorylated (Dorée and Galas, 1994). The cdk-activating kinase, CAK, is responsible for the phosphorylation of Thr 161 (Clarke, 1995; Solomon et al., 1992) and is itself a complex of cdk7 and cyclin H (Fischer and Morgan, 1994). Its activity also requires phosphorylation at an equivalent threonine residue (Thr 176), suggesting that another kinase may lie upstream of CAK in the pathway (Clarke, 1995).

1.2.2 Inactivation of cdk complexes

There are three different mechanisms to inactivate cdks. The first one involves inhibitory phosphorylation of the catalytic subunit. Cdc2 is inhibited by phosphorylation of Thr 14 and Tyr 15 and at mitosis these sites are dephosphorylated. In fission yeast, the product of the wee1 gene phosphorylates Tyr 15, while the product of the cdc25 gene dephosphorylates Thr 14 and Tyr 15 to produce an active cdc2 (Coleman and Dunphy, 1994). The main purpose of this phosphorylation is to regulate the timing of the activation of the kinase (Dorée and Galas, 1994). For cdk2, the same amino acids are phosphorylated, but no consequences on cdk2 functions have so far been described (Gu et al., 1992). Furthermore, no other studies have shown that other cdks are similarly phosphorylated.

The second mechanism primarily acts on the G1/S cdk-cyclin complexes. In mammalian cells, low molecular weight CDIs bind either to the catalytic subunit, the cyclin or the cdk-cyclin complex and inhibit cdk

activity. At present, in mammalian cells, three CDIs have been identified and are called p21, p27 and p16 accordingly to their molecular weights. p21, also called Cip1, $p20^{cap1}$ and Waf1, is the best studied CDI (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). *In vivo*, p21 is associated with many cdk-cyclin complexes, including cdk2-cyclin A/E and cdk4-cyclin D. *In vitro*, p21 is a potent inhibitor of cdk2 and cdk4 (Ki<10 nM) (Elledge and Harper, 1994). Furthermore, p21 plays a role in the p53-mediated G1 checkpoint by inhibiting cdks and blocking DNA replication (see later discussion). p27 directly inhibits cdk2-cyclin E and cdk4-cyclin D complexes and plays a role in the G1 arrest in response to extracellular signals, such as transforming growth factor. Unlike p21 and p27, p16 appears to be a selective inhibitor of cdk4 and cdk6 that are activated by D-type cyclins (Elledge and Harper, 1994).

The third mechanism for inactivating cdks is proteolysis of the cyclin subunit. Cyclin levels normally oscillate during the cell cycle and were first observed in sea urchin eggs being destroyed at each cleavage division (Evans et al., 1983). Cyclin proteolysis makes Thr 161 of cdc2 (or equivalent residues in other cdks) accessible to phosphatases that inactivate the catalytic subunit (Dorée and Galas, 1994). The crystal structure of the cyclinA-cdk2 complex has recently been solved and the cyclin A binding causes conformational changes that realign the active site residues (Jeffrey et al., 1995). The mechanism of cyclin proteolysis is well understood for the mitotic cyclins and is discussed in detail in section 1.3.3. In budding yeast, a PEST-rich carboxyl-terminal region confers instability to G1 cyclins, which are rapidly turned over. However, the mechanism of degradation of G1 cyclins in yeast and mammalians cells is still unknown.

1.2.3 Role of cdks in the cell cycle

The main function of cdks is to control cell cycle progression by phosphorylating various substrates. The substrate specificity of the different cdks is quite similar and includes a proline residue carboxyl terminal to the phosphorylation site (Dorée and Galas, 1994). Therefore, it is assumed that temporal expression, spatial distribution and the specific cyclin partners are responsible for targetting cdks to their specific substrates (Nigg, 1993).

The major cdks that are essential for the transition from G1 to S phase are cdk2 and cdk3. When inactive mutants of cdk2 and cdk3 were overexpressed in cells, the cells arrested in G1 (Dorée and Galas, 1994). The other cdks were not found to be required for this transition. One of the main cdk substrates which controls G1/S progression is the tumour suppressor, retinoblastoma protein (Rb). Rb stops the cell cycle by binding to different transcription factors, such as E2F, and limiting the potential to transactivate genes required for progression through G1 (Sherr, 1994). Hyperphosphorylation of Rb on specific cdk sites at late G1 cancels the growth suppressive function and allows cell cycle progression. Thus cdks are important for the G1/S transition.

Another cellular process affected by cdks is DNA replication. Cdk2-cyclin A is activated before the onset of DNA replication, associates with the replication foci and is necessary for DNA replication (Nigg, 1993). Some possible substrates include transcription factors involved in the G1/S phase transition and the single-stranded DNA binding factor RPA. However, the effects of phosphorylation on these proteins are unknown.

Another major function of cdks is the control of mitosis, which is discussed in detail below as it is central to my research project.

1.3 Mitosis

The entry and exit from mitosis is controlled by the maturation-promoting factor (MPF), a complex of cdc2 and either cyclin A or cyclin B (Coleman and Dunphy, 1994; King et al., 1994). MPF is the dominant regulatory factor that phosphorylates a variety of structural and regulatory proteins during mitosis. The levels of cyclins A and B oscillate during the cell cycle, reaching a maximum during G2 phase and mitosis when MPF is optimally active (Gu et al., 1992; King et al., 1994). Exit from mitosis and entry into interphase requires the inactivation of MPF through the degradation of cyclins near the end of metaphase (Murray et al., 1989).

1.3.1 Mitotic Control by cdc2

Major events associated with entry into mitosis in higher eukaryotes include chromosome condensation, formation of the mitotic spindle, nuclear envelope breakdown and considerable changes in the cytoskeleton. Chromosome condensation is accompanied by the hyperphosphorylation of histones H1 and H3 and other non-histone, chromatin-associated proteins. Furthermore, the dissassembly of the nucleoli and repression of transcription involve the phosphorylation of major nucleolar proteins and transcription factors. Cdc2 has been shown to be involved in many of these effects.

Originally, cdc2 was thought to act as the master regulator by activating downstream kinases. However, few kinases proved to be potential substrates of cdc2-cyclin B. These include $p60^{src}$, $p150^{abl}$, casein kinase II and MAP kinase (King et al., 1994). Several structural proteins have been identified as likely direct targets of cdc2 (Nigg, 1993). Specifically, cdc2-cyclin B phosphorylates histone H1 and weakens its association with DNA, which may allow access for other putative factors responsible for chromosome

condensation (Th'ng et al., 1990). Furthermore, cdc2 is responsible for nuclear envelope breakdown by phosphorylating lamins directly and most likely also the lamin B receptor of the inner nuclear membrane (Heald and Mckeon, 1990). In addition to lamins, other cytoskeletal intermediate filaments such as vimentin undergo structural reorganization and are phosphorylated by cdc2 (Chou et al., 1989; Chou et al., 1990). Cdc2 may also cause nucleolar disassembly by phosphorylating structural proteins such as nucleolin (Peter et al., 1990) and may regulate mitotic reorganization of microtubule and microfilament networks (Tombes et al., 1991; Satterwhite and Pollard, 1992). Since cdc2 directly affects so many structural substrates involved in mitosis, it is more appropriate to regard it as a workhorse rather than a master regulator.

Within the last year, studies have indicated the existence of kinases that may be downstream from cdc2 or may be independent of it. Using protein phosphatase inhibitors and a cell line with a thermosensitive cdc2 gene product, it was shown that chromosome condensation can occur without cdc2 activity and histone H1 phosphorylation (Guo et al., 1995). This suggests that another kinase possibly independent of the cdc2 pathway may be responsible for some mitotic events. In *Aspergillus nidulans*, the NIMA kinase is essential for entry into mitosis. The forced expression of NIMA without cdc2 activity causes mitotic chromosome condensation and the activity of NIMA is under the control of and phosphorylated by cdc2-cyclin B (Pu and Osmani, 1995; Ye et al., 1995). NIMA is downstream of the cdc2-cyclin B complex. It is thought that cells coordinately activate both cdc2-cyclin B and NIMA to initiate mitosis and that both kinases activate each other's functions (Ye et al., 1995). Further studies have shown the existence of a NIMA-like pathway in vertebrate cells (Lu and Hunter, 1995) and a NIMA-like homologue Nek2 in vertebrate cells (Fry et al., 1995; Schultz et al., 1994).

Along with cdc2, the NIMA kinase and other undiscovered kinases may also play important roles in the regulation of mitosis.

1.3.2 Regulation of cdc2 by phosphorylation

As described briefly in Section 1.2.1, phosphorylation of conserved residues plays an important role in the regulation of cdc2. The phosphorylation of Thr 161 is necessary for cdc2 activation and is mediated by CAK (Fig. 2). Protein phosphatase 2A (PP2A) is thought to oppose the action of CAK (Lee et al., 1991), since the addition of PP2A to *Xenopus* egg extracts inhibits Thr 161 phosphorylation (Lee et al., 1994). However, it is not known whether PP2A acts directly on cdc2, indirectly on CAK, or on a Thr 161-directed phosphatase.

In contrast, phosphorylation on either Thr 14 or Tyr 15 inhibits cdc2 activity, because these sites overlap the ATP-binding site (Fig. 2). Wee1 kinase phosphorylates cdc2 on Tyr 15 and another unknown inhibitory kinase phosphorylates on Thr 14 (Dunphy, 1994). Tyrosine dephosphorylation by cdc25, a specific and highly regulated tyrosine phosphatase, is necessary for the activation of cdc2. Furthermore, cdc25 phosphatase and wee1 kinase are regulated by other kinase-phosphatase systems and even by cdc2 itself. Cdc25 is weakly active during interphase and becomes highly active at the G2-M transition due to phosphorylation at the N-terminal regulatory domain by cdc2 and/or MPM-2 epitope (ME) kinases (Dunphy, 1994). One ME kinase was identified as a mitogen-activated protein (MAP) kinase (Ferrell et al., 1991; Minshull et al., 1994). The inhibitory phosphatase affecting cdc25 is similar to PP2A.

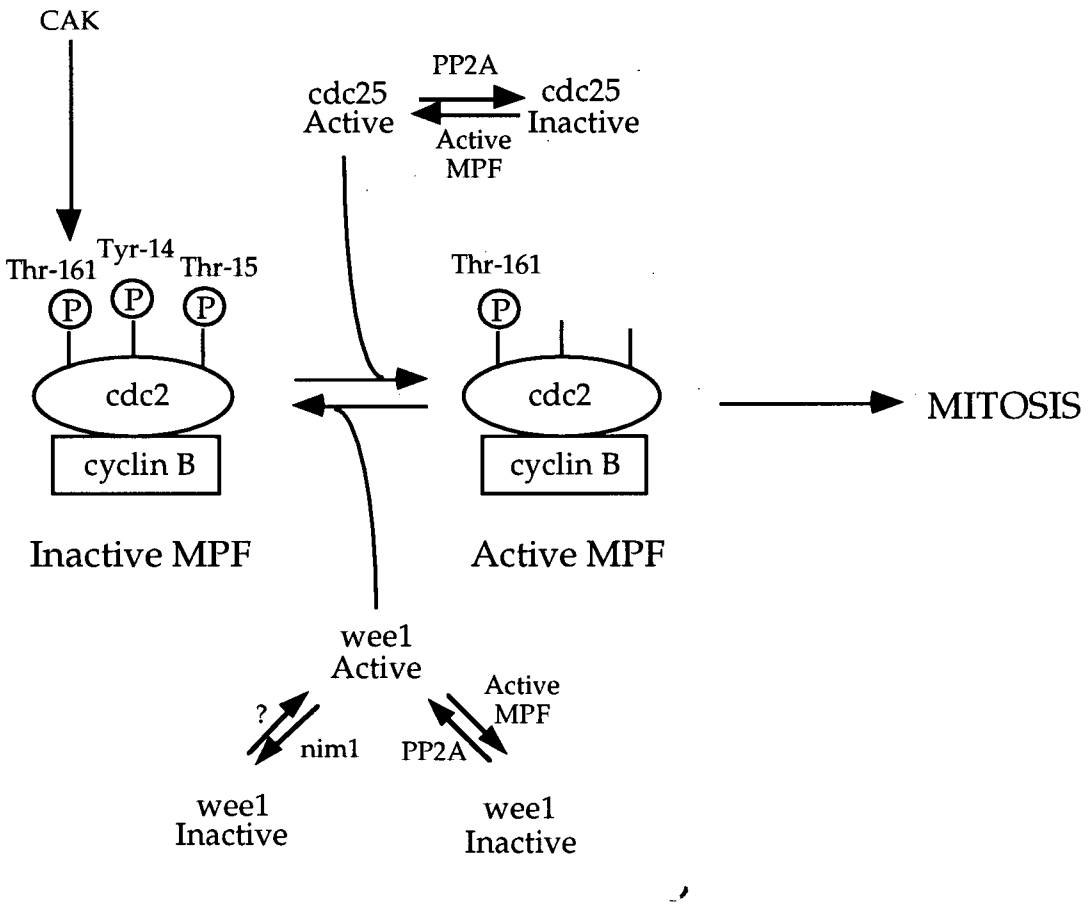


Figure 2. The regulation of MPF activity.

MPF is required for entry and exit from mitosis and is a complex between cdc2 and its regulatory subunit, cyclin B. MPF activity is controlled by the phosphorylation of specific amino acid residues, Thr-14, Tyr-15 and Thr-141. Their phosphorylation state is controlled by wee1 kinase, CAK and cdc25 phosphatase.

Wee1 is underphosphorylated during interphase and is able to phosphorylate cdc2 exclusively on Tyr 15, but is hyperphosphorylated and nearly inactive at mitosis (Fig. 2). In yeast, the nim1 kinase phosphorylates wee1 at the carboxyl-terminal end to inactivate it, but a homologous kinase in mammalian cells has not been found (Coleman and Dunphy, 1994). Another kinase-phosphatase system phosphorylates the amino-terminal region of wee1 and inactivates it. Potential kinase candidates include MPF and ME kinases and the phosphatase that maintains wee1 in its active state during interphase has characteristics similar to PP2A.

There are some interesting parallels between the regulation of wee1 and cdc25. Both proteins are extensively phosphorylated near the amino-terminal regions at the G2-M transition and are underphosphorylated in interphase. PP2A-like phosphatases, cdc2 and ME kinases are implicated in both cdc25 and wee1 regulation. It seems that a similar kinase-phosphatase system regulates both proteins.

1.3.3 Cyclin Accumulation and Proteolysis

The activity of MPF oscillates in the cell cycle and controls mitosis. Since cdc2 is constitutively present within the cells, the accumulation and periodic destruction of cyclins determines the oscillation of MPF activity (Fig. 3). The mitotic cycle can be divided into three transitions: 1) the accumulation of cyclin B activates MPF and initiates prophase; 2) MPF activates a proteolytic system that causes cyclin B destruction and initiation of anaphase; and 3) the destruction machinery is shut off and the cell cycle is reset (King et al., 1994).

Cyclin B levels must reach a threshold for cells to enter mitosis, but once this level is attained, a lag period follows during which all the

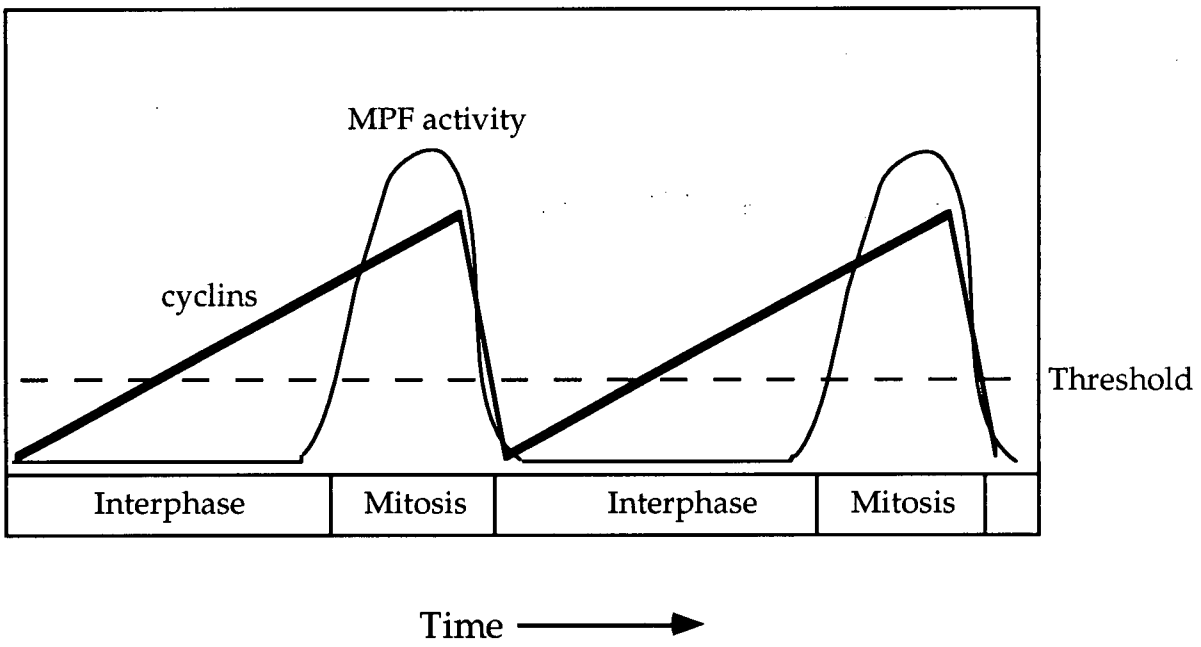


Figure 3. The activity of MPF and levels of cyclins vary through the cell cycle.

The activity of MPF oscillates throughout the cell cycle reaching maximum levels at mitosis (solid lines). The cell varies the MPF activity by varying the levels of cyclins, the regulatory subunits. Cyclins slowly build up from the beginning of interphase, reaching maximum levels at mitosis and are rapidly degraded at the end of mitosis (bold lines). The minimal threshold level of cyclins necessary for entry into mitosis is shown in broken lines (King et al., 1994).

components are present, but MPF is inactive (Murray et al., 1989; Solomon et al., 1990). This implies other positive and negative regulatory factors, including phosphorylation of Thr 161 and dephosphorylation of Thr 14 and Tyr 15 (Fig. 2). It seems that tyrosine dephosphorylation plays the most important role in the regulation of cdc2 activity. However, it is likely that other mitotic regulators linked to the central MPF activation can limit mitosis in different circumstances. They may include kinases such as NIMA kinase, ME kinases, calmodulin-dependent kinases and MAP kinases (King et al., 1994) and other phosphatases.

After the condensed chromosomes have been aligned at the metaphase plate, it is thought that MPF activates the cyclin degradation pathway, which then induces anaphase and inactivation of MPF. Degradation requires cyclins A and B to be bound to cdc2 (Stewart et al., 1994; van der Velden and Lohka, 1994) and also requires protein kinase activity, possibly cdc2-cyclin B itself since cdc2-cyclin B can trigger cyclin degradation when added to cell-free extracts of interphase *Xenopus* eggs (Felix et al., 1990). Yet cdc2 does not seem to phosphorylate cyclin B directly because mutation of the major cdc2 phosphorylation sites in cyclins has no effect on their degradation (Izumi and Maller, 1991). Rather, cdc2 is thought to phosphorylate another unidentified protein or kinase which in turn can activate cyclin degradation (Felix et al., 1990). Recent studies have shown that protein kinases besides cdc2 can trigger cyclin degradation (Watanabe et al., 1991; Holloway et al., 1993; Lorca et al., 1994; Lorca et al., 1993; Morin et al., 1994). Treatment of cells with okadaic acid, a known protein phosphatase 1 and 2A inhibitor, activates cyclin degradation in the absence of cdc2 activity, but requires the activity of a protein kinase (Guo et al., 1995). In *Xenopus* egg extracts, Ca2+/calmodulin-dependent protein kinase II can activate this degradation pathway (Holloway

et al., 1993; Lorca et al., 1994; Lorca et al., 1993; Morin et al., 1994). Another kinase, c-mos, prevents proteolytic degradation of cyclins and arrests unfertilized eggs at the second meiotic metaphase (Sagata et al., 1989). Upon fertilization, a calcium-dependent process inactivates c-mos resulting in cyclin degradation (Watanabe et al., 1991). In mammalian cells, the other kinases involved in cyclin proteolysis are unknown. The dependency of the cyclin degradation process on protein phosphorylation suggests that protein phosphatases also play a role in cyclin degradation. One study already showed that an okadaic acid-sensitive phosphatase negatively controls cyclin degradation in amphibian egg extracts (Lorca et al., 1991).

Cyclin degradation near the end of mitosis occurs through the ubiquitin-dependent proteolytic pathway, which involves the conjugation of a small protein, ubiquitin, to other proteins that are then targeted to be degraded by a large multicatalytic protease called the proteasome (Ciechanover and Schwartz, 1994; Glotzer et al., 1991; Murray, 1995). Other components of this system include the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). The pathway can be summarized as follows: E1 activates the ubiquitin molecule, then transfers it to E2 and with the help of E3 for substrate recognition, E2 donates the ubiquitin to protein substrates. Then the proteasome degrades the tagged proteins.

Cyclin B is ubiquitinated in mitotic, but not in interphase *Xenopus* egg extracts and ubiquitination is dependent on the presence of the intact destruction box, a short conserved sequence (RAALGNISEN) at the cyclin N-terminus, which when mutated stabilizes cyclins against proteolysis (Glotzer et al., 1991; Luca et al., 1991). The destruction box does not serve as a ubiquitination site and may be involved as a recognition site for an enzyme

in this pathway. The cyclin protein can be divided into two domains, the N-terminus, which targets the protein for degradation and the C-terminus, which is required for binding and activation of cdc2. In yeast, cyclin degradation and exit from mitosis require a specific ubiquitin-conjugating enzyme and a functional proteasome (Ghislain et al., 1993; Seufert et al., 1995). There are two different hypotheses about the mechanism of cyclin degradation: cyclin is modified and converted to a form that is susceptible to ubiquitin ligase or a cyclin-specific ligase is activated during mitosis. The second possibility seems more valid since cdc2 is thought to phosphorylate components of the ubiquitin-proteasome pathway, possibly specific ubiquitin-cyclin ligases (Hershko et al., 1994; King et al., 1995; Sudakin et al., 1995). This would link the cell cycle machinery to the ubiquitin-dependent degradation pathway. Ongoing research will further our understanding of the cyclin degradation pathway.

1.4 Cell Cycle Checkpoints

In order for a cell to successfully survive and propagate, the cell cycle must be a tightly controlled, ordered process. Cellular feedback mechanisms, called cell cycle checkpoints, monitor the completion of earlier cellular events before allowing the initiation of later ones. These checkpoints ensure the proper division of a cell into two daughter cells and defects in these checkpoints result in dead, aneuploid or mutant cells. In multicellular organisms, aneuploidy and mutation can produce uncontrolled cell proliferation, which gives rise to cancer. Cell cycle checkpoints involve the whole process of monitoring cell cycle events, generating signals in response to the errors and then halting the cell cycle at specific points. Two important features of checkpoints include the amplification of the initial signal

generated by a small number of damaged molecules to a level that can halt the cell cycle.and a rapid response to stop the cell cycle before irreversible damage is done to the genome. The three major checkpoints presently studied are: 1) G1 checkpoint, when cells will not enter S-phase with damaged DNA; 2) G2 checkpoint, when the cell will not enter mitosis with unreplicated or damaged DNA; and 3) spindle asssembly checkpoint, when the cell will not exit mitosis with improperly formed spindles (Fig. 4) (Murray, 1994).

In the past year, a major advance linking the cell cycle with cell cycle checkpoints has been discovered. It appears that CDIs are important for the G1 arrest induced by DNA damage. Specifically, when DNA damage occurs, there is an increase in the level of the tumour suppressor protein p53. p53 is a transcriptional activator of the p21 CDI gene. Therefore, increased levels of p53 induce p21 transcription. p21 interacts directly with and inhibits the cdk-cyclin complexes that are required for the progression of the cell cycle and DNA replication (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993). Thus, the cell cycle is halted at the G1 checkpoint in response to damaged DNA until the DNA is repaired. The G2 checkpoint responds to damaged or unreplicated DNA through two pathways, which share some common features. Some studies in yeast and mammalian cells suggest that the G2 checkpoint induced by unreplicated DNA prevents the dephosphorylation of Tyr 15 and therefore, the activation of cdc2 (Enoch and Nurse, 1991; Smythe and Newport, 1992). Furthermore, preventing the phosphorylation of Tyr 15 allows cells that have unreplicated DNA to enter mitosis (Smythe and Newport, 1992). Although there have been major recent advances in cell cycle checkpoint research, there are many questions still to be answered. For

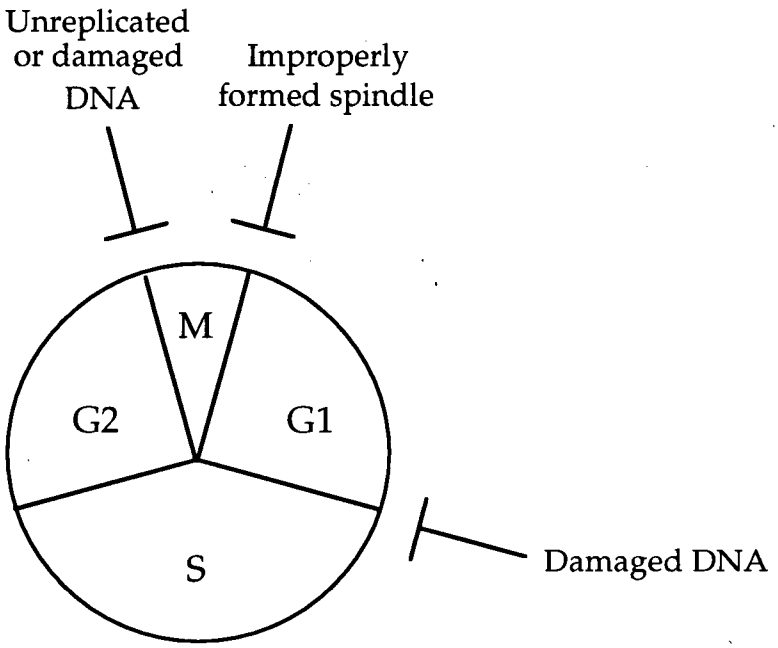


Figure 4. Eukaryotic cell cycle checkpoints.

Checkpoints are feedback mechanisms that monitor the completion of earlier events before allowing the initiation of later ones. The three major checkpoints include the G1 checkpoint, the G2 checkpoint and the spindle assembly checkpoint.

example, little is known about the mechanisms by which checkpoints monitor events and arrest the cell cycle machinery.

Major characteristics of cancer cells are increased genomic instability and the accumulation of several independent mutations. In principle, lesions in cell cycle checkpoint genes would increase the chance of genetic damage and consequently, cancer (Hartwell, 1992; Li and Deshaies, 1993; Murray, 1992). Cancer cells do indeed have weaker checkpoint controls (Hartwell, 1992; Murray, 1992). Most chemotherapeutic agents affect some process involving DNA, such as blocking DNA replication or damaging DNA. Since many tumour cells have weaker checkpoint controls, checkpoint inhibitor drugs may increase the selectivity and cytotoxicity of other chemotherapeutic drugs. Ideally normal cells would not be affected by the combination of drugs while the tumour cells with weaker feedback controls would be killed more effectively. Therefore, the study of the cell cycle and its checkpoints is quite relevant to modern cancer research and may provide novel methods in cancer therapy.

1.5 Serine/Threonine Protein Phosphatases

The reversible phosphorylation of proteins has emerged as a fundamental mechanism for the control of many cellular functions, such as metabolic systems, cell division, muscle contraction, gene expression and cellular differentiation. Approximately one-third of cellular proteins are thought to undergo reversible phosphorylation (Depaoli-Roach et al., 1994). This post-translational modification alters the properties of key regulatory proteins involved in specific cellular pathways. To date, a few hundred kinases have been identified and characterized, but only a limited number of protein phosphatases have been identified. The regulation and the

physiological roles of these phosphatases are not well understood. Our laboratory is interested in the role of protein phosphatases, especially serine/threonine phosphatases, in the cell cycle and cell division.

1.5.1 Classes of Serine/Threonine Phosphatases

The serine/threonine phosphatases can be divided into two categories, type 1 and type 2 (Ingebritsen and Cohen, 1983) (Table 2). Type 1 phosphatase catalytic subunits are inhibited by two protein inhibitors, inhibitor-1 and inhibitor-2 and dephosphorylate the β subunit of phosphorylase kinase. Type 2 phosphatases are insensitive to the inhibitors and preferentially dephosphorylate the α subunit of phosphorylase kinase. The type 2 phosphatases can be further divided into three classes: PP2A, 2B and 2C.

Table 2. Classification of Ser/Thr Protein Phosphatases

	Catalytic Subunit	Regulatory Subunit
TYPE 1[a] ATP-Mg dependent Glycogen/SR-associated Myofibril-associated Nuclear	C1 α, γ1, γ2, δ	 R_{1-2} R_{GL} R_{MY} NIPP-1
TYPE 2A Phosphatase 2A2 Phosphatase 2A1 Phosphatase 2A0 Phosphatase PCS_M	C2 α, β	 A α, β B α, β, γ B'α, β, γ, δ B "
TYPE 2B Calcineurin	A α, β, γ	 B calmodulin
TYPE 2C Mg-activated	C α, β	none

[a]ref: (Depaoli-Roach et al., 1994; Mayer-Jaekel and Hemmings, 1994)

Unlike PP2A, PP2B and 2C require Ca^{2+} and Mg^{2+} for their activity. Both types consist of a catalytic subunit bound to at least one regulatory subunit, which serves to specify the function of the enzyme. The catalytic subunits, except for PP2C, belong to a single gene family and there is a high degree of homology between PP1 and PP2A catalytic subunits from yeast to mammals.

1.5.2 PP1 and PP2A inhibitors

Several non-protein inhibitors of protein phosphatases have been discovered. Okadaic acid, the most commonly used, is produced by marine dinoflagellates and inhibits protein phosphatases in the following order PP2A>>PP1>> PP2B (Bialojan and Takai, 1988; Cohen et al., 1990). Other inhibitors include tautomycin, calyculin A and the microcystins (Holmes and Boland, 1993) (Table 3). Because okadaic acid and calyculin A are cell-permeable, they have been used extensively to study protein phosphorylation *in vivo*. However, the discrimination between PP1 and PP2A *in vivo* by these inhibitors is not the same as *in vitro*. For okadaic acid, the IC_{50} for PP2A is 0.2 nM, 100-fold lower than for PP1, but the higher concentrations needed *in vivo*, in the range of 1 μM, may well inhibit both PP1 and PP2A.

Table 3- IC_{50} values of protein phosphatase inhibitors

Inhibitors	Phosphatase Inhibition IC_{50} (nM)[a]	
	PP1	PP2A
Okadaic acid	2.0	0.2
Tautomycin	0.2	1.0
Calyculin A	0.5-2	0.1-1
Microcystin-LR	0.15	0.15
Inhibitor-1	0.45	-
Inhibitor-2	0.8	-

[a] Ref: (Holmes and Boland, 1993)

1.5.3 Physiological Roles of PP1 and PP2A

PP1 and PP2A activities represent a significant proportion of the phosphatase activity within cells (Depaoli-Roach et al., 1994). Therefore, it is difficult to discuss all their physiological roles within the cell. The study of the role of PP1 and PP2A in the control of metabolic processes has been intensive. PP1 is involved in glycogen metabolism, calcium transport and muscle contraction (Hubbard and Cohen, 1993). In signal transduction pathways initiated by growth factors, a role of PP2A has been suggested. Some of the kinases, such as S6 kinase and ERK kinases, can be inactivated by PP2A *in vitro* and the treatment of cells with protein phosphatase inhibitors causes a marked activation of these kinases (Ballou et al., 1988; Gomez and Cohen, 1991; Gotoh et al., 1990).

Biochemical studies have shown that PP1 and PP2A play important roles in the cell cycle by controlling the activity of cdc2 and its substrates (refer to section 1.3). Treatment of fibroblasts with okadaic acid results in increased levels of phosphorylation of proteins such as Rb, p53 and nucleolin, some of which are cdc2 substrates. Furthermore, treatment of BHK cells and *Xenopus* oocyte extracts with okadaic acid caused premature mitosis and the activation of histone H1 kinase (Goris et al., 1989; Yamashita et al., 1990). In *Xenopus* oocytes, one group identified a PP2A-like phosphatase, INH, that inhibits active MPF by specifically targetting Thr-161 (Lee et al., 1991). As mentioned before, cdc25 phosphatase is a substrate of PP1 and PP2A *in vitro*. The microinjection of fibroblasts with anti-PP1 antibodies blocked completion of mitosis and the microinjection of PP1 catalytic subunits accelerated mitosis (Fernandez et al., 1992). Further work with *Xenopus* egg extracts showed that PP1 activity increases at the onset of both S phase and mitosis (Walker et al., 1992). Finally, genetic studies with *S. pombe, S. cerevisiae* and *Drosophila*

have determined that disruptions or mutations in catalytic subunits of protein phosphatases causes lethality or premature mitosis (Depaoli-Roach et al., 1994; Kinoshita et al., 1990).

1.6 The Antitumour Drug Fostriecin

Fostriecin is a structurally novel antitumour drug isolated from a streptomycete (Stampwala et al., 1983; Tunac et al., 1983). It was shown to be active against leukemia cells as well as lung, breast and colon cancer cells *in vitro* and experimental tumours in mice (Jackson et al., 1985; Leopold et al., 1984; Scheithauer et al., 1986). It is now in phase I clinical trials in the United States and Canada (Moore et al., 1995; Schilsky et al., 1994). Since the intracellular target of fostriecin is unknown, my project was concerned with studying its effects and elucidating its mechanism of action.

1.6.1 Historical Perspective

In screening novel antitumour compounds, the fermentation beer of an actinomycete isolate was observed to inhibit the growth of L1210 mouse leukemia cells and also to possess antileukemic activity in mice (Jackson et al., 1985). The culture was isolated from a Brazilian soil sample and the organism was identified as *Streptomyes pulveraceus* subspecies *fostreus* (Tunac et al., 1983). Three compounds were isolated and characterized in the fermentation beer. The most potent compound was initially designated as CI-920 (Stampwala et al., 1983) and later, fostriecin (Scheithauer et al., 1986). Fostriecin is a water-soluble phosphate ester with a conjugated triene system (Fig. 5). Structure-activity experiments have shown that the primary alcohol moiety has no effect on fostriecin's activity. However, the removal of the phosphate ester or the opening of the lactone ring reduces the activity considerably (Jackson et al., 1985).

The potency of fostriecin varies among different types of cancer. Fostriecin is particularly active against L1210 leukemia and HCT-8 cell lines (Jackson et al., 1985; Tunac et al., 1983). In ACR mice, fostriecin is especially effective against L1210, P388 leukemias and B16 melanoma and has slight activity against colon carcinoma 38, M5076 sarcoma and mammary adenocarcinoma *in vivo*. However, fostriecin is inactive against Lewis lung tumour, LC12 lung tumour, CD8F1 mammary carcinoma and Ridgeway osteogenic sarcoma (Jackson et al., 1985; Leopold et al., 1984). Further studies using a human tumour cloning assay determined that fostriecin has effects on ovarian cancer, breast and human lung cancer cells *in vitro* (Scheithauer et al., 1986). Overall, fostriecin showed enough promise as an antitumour drug for phase I clinical trials to be initiated.

Biochemical studies showed that fostriecin causes the inhibition of RNA and DNA synthesis in intact L1210 cells and also of protein synthesis at higher drug concentrations (Fry et al., 1984; Jackson et al., 1985; Leopold et al., 1984). However, fostriecin does not cause DNA strand breakage, nor does it inhibit RNA polymerase or DNA polymerase directly or deplete nucleotide pools (Fry et al., 1984; Jackson et al., 1985; Leopold et al., 1984). Flow cytometry studies showed that growth-inhibitory levels of fostriecin cause accumulation of cells in the G2/M phase and that higher drug concentrations cause an S phase block (Jackson et al., 1985). Fostriecin is also an inhibitor and irreversible inactivator of the reduced folate membrane transport system and appears to enter cells through this receptor (Fry et al., 1984; Jackson et al., 1985). This may explain fostriecin's limited antitumour spectrum, since it is inactive against cells with low levels of the reduced folate receptor. However, a recent study has disputed this possibility (de Jong et al., 1991) and the mechanism of entry should be considered uncertain.

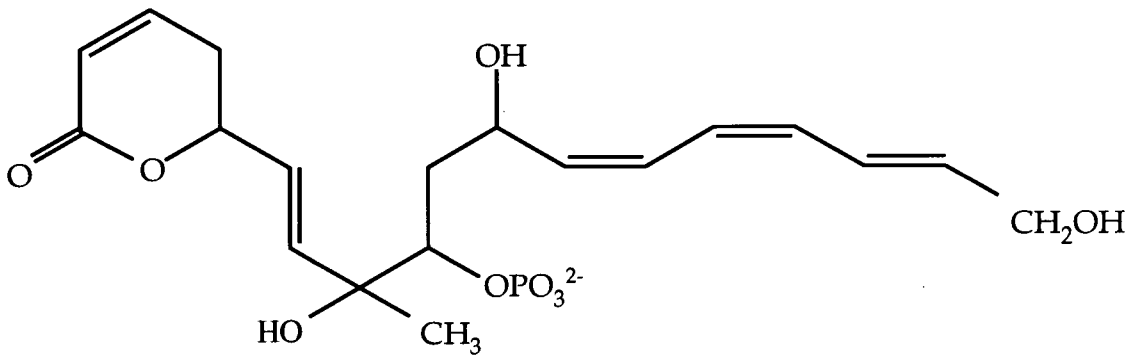


Figure 5. The Structure of Fostriecin

Fostriecin is a structurally novel antibiotic isolated from *Streptomyces pulveraceus*. It displays antitumour activity against leukemia cells as well as lung, breast and colon cancer cells. The chemical nomenclature is 2H-pyran-2-one, 5-6-dihydro-6-[3,6,13-trihydroxy-3-methyl-4-(phosphonoxy)-1, 7, 9, 11-tridecatetraenyl].

In more recent studies, fostriecin was found to be an inhibitor of purified DNA topoisomerase II *in vitro* with a IC_{50} of 40 μM (Boritzki et al., 1988; Gedik and Collins, 1990), but fostriecin did not have any effects on this enzyme in cell extracts (Frosina and Rossi, 1992). Furthermore, another study showed that fostriecin does not inhibit VM-26 mediated DNA-topoisomerase II complexes in living cells, suggesting that topoisomerase II is not a target of fostriecin *in vivo* (Chen and Beck, 1993).

1.6.2 Inhibition of protein phosphatases 1 and 2A

Some drugs that target topoisomerases stabilize a covalent link between topoisomerase and DNA via a single-stranded or double stranded DNA break. The cells treated with these drugs arrest in G1 or G2 phase because of DNA lesions. Initially, we wanted to determine if fostriecin induced G2 arrest, but we found instead that fostriecin-treated cells were accelerated into mitosis, but were unable to exit mitosis (Roberge et al., 1994). Many morphological characteristics of early mitosis were seen, such as rounding up of cells, chromosome condensation, nuclear lamina depolymerisation and separation of spindle poles, but the later events such as metaphase alignment of the chromosomes, separation and segregation of sister chromatids and cytokinesis were rarely observed.

Further experiments showed that fostriecin forces entry into mitosis even with cells arrested at the G2 checkpoint (Roberge et al., 1994). The cells were arrested in S-phase with incompletely replicated DNA by the DNA polymerase inhibitor aphidicolin or in G2 phase with the topoisomerase I inhibitor campothecin or the topoisomerase II inhibitor VM-26. Treatment with fostriecin allowed cells to overcome the G2 checkpoint block.

Other chemicals are known to cause premature appearance of mitotic events or to overcome the G2 checkpoint. One example is the protein phosphatase 1 and 2A inhibitor okadaic acid, which can cause premature mitosis (Ghosh et al., 1992; Yamashita et al., 1990). Using an *in vitro* protein phosphatase assay, fostriecin was also found to inhibit protein phosphatase 1 with an IC_{50} of 4 μM and protein phosphatase 2A with an IC_{50} of 40 nM (Roberge et al., 1994). Since fostriecin inhibits protein phosphatase 2A 1000-fold and protein phosphatase 1 10-fold more potently than topoisomerase II (IC_{50}=40 μM), protein phosphatases 1 and 2A are more likely targets of fostriecin than topoisomerase II. These results suggest that fostriecin acts directly on the catalytic subunits of protein phosphatases.

In another study, fostriecin was found to induce chromosome condensation in the absence of cdc2 activity and histone H1 hyperphosphorylation (Guo et al., 1995). Chromosome condensation in normal mitosis is accompanied by phosphorylation of histone H1 and activation of cdc2. However, chromosome condensation caused by fostriecin does not require cdc2 activity or histone H1 phosphorylation as demonstrated by using FT210 cells, which have a temperature sensitive lesion in the cdc2 gene. Instead, histone H2A and histone H3A become hyperphosphorylated in response to fostriecin. By uncoupling the process of chromosome condensation from cdc2 activity and H1 phosphorylation, fostriecin was used as a tool to further study the processes of chromosome condensation.

1.6.3 Research objectives

My project's objectives can be divided into two major parts: finding the intracellular target of fostriecin and studying the effects of fostriecin on the cell cycle. Since fostriecin is a potent *in vitro* inhibitor of protein

phosphatase, we wanted to determine whether a protein phosphatase is the *in vivo* target. As a protein phosphatase inhibitor, fostriecin should have effects on the phosphorylation of various proteins within the cell. We discovered a highly phosphorylated 55 kDa protein, identified the protein to be the intermediate filament vimentin and further studied the effects of this phosphorylation. Other known protein phosphatases, such as okadaic acid and calyculin A, cause very similar effects, strongly suggesting that fostriecin is a protein phosphatase inhibitor *in vivo*.

The second objective was to study the effects of fostriecin on the cell cycle. In the study by Guo et al., 1995, fostriecin was found to induce the degradation of cyclin A and cyclin B in mitotic FT210 cells. We wanted to determine whether cyclins were also degraded in human cancer cell lines, such as the human cervix epitheloid carcinoma HeLa cell line or lymphoma Jurkat cell line. We found that fostriecin induces the premature degradation of cyclins A and B and further characterized these effects. Fostriecin-induced cyclin degradation is dependent on staurosporine-sensitive kinases and the ubiquitin-dependent proteasome pathway. Since cyclins are essential for the progression of the cell cycle, fostriecin's ability to induce cyclin degradation may be important for its cytotoxic effects. Instead we found that the cytotoxicity was not dependent on cyclin degradation, but partially dependent on the degradation of other proteins.

Chapter 2

Materials and Methods

2.1 Cell Culture

Baby hamster kidney-21 (BHK-21) cells were grown as monolayers in Dulbecco's minimal essential medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) and antibiotics at 37°C in humidified 10% CO_2. The human cervix epitheloid carcinoma HeLa cells were grown as monolayers and human lymphoma Jurkat cells as a suspension in RPMI-1640 supplemented with 10% FBS and antibiotics at 37°C in 5% humidified CO_2. The mouse mammary carcinoma FT210 cells were grown in suspension in RPMI-1640 supplemented with 10% FBS and antibiotics at 32°C in humidified 5% CO_2. The HeLa, Jurkat and FT210 cells were first synchronized by incubation in isoleucine-free RPMI containing 10% dialyzed, heat-inactivated FBS for 16 h, followed by a 9 h incubation in normal RPMI medium containing 2.5 μg/ml aphidicolin. The HeLa and Jurkat cells were then either released from the S phase block for 1-2 h and the drugs were then added or they were released from the aphidicolin block in fresh medium containing 0.4 μg/ml nocadazole for 12 h to arrest cells in mitosis. FT210 cells were released from the aphidicolin block into normal medium at 39°C for 18 h to arrest cells in G2 phase.

2.2 Drug Treatment

Fostriecin was obtained from the NCI (NSC 339638, 94528) in vials containing 25 mg fostreicin, 39 mg ascorbic acid to act as an antioxidant and NaOH to neutralize to pH 7.0. It was added to the cells as a fresh stock

solution in phosphate-buffered saline. Ascorbic acid was used from a stock that was adjusted to pH 7.0. Okadaic acid (Gibco) was used from a 0.5 mM stock in 10% DMSO and staurosporine (Sigma) was from a 5 μg/ml stock in DMSO. The protease inhibitors N-acetyl-leu-leu-norleucinal (LnLL) (Sigma), N-benzyloxycarbonyl-Ile-Glu(*O-t-* butyl)-Ala-leucinal (PSI), and N-benzyloxycarbonyl-Leu-Leucinal (CLL) (kindly donated by S. Wilks) were used from a 25 mM stock in DMSO.

2.3 Western Blotting

Western blotting was performed as described in Guo et al. (1995) with minor modifications. Briefly, after drug treatment, the cells were harvested and lysed on ice by pipetting in lysis buffer (100 mM Tris, pH 8.0, 250 mM NaCl, 10 mM $CaCl_2$, 0.5% Triton X-100, 1 mM PMSF, 50 μg/ml aprotonin, 50 μg/ml leupeptin, 20 μg/ml pepstatin, 100 U/ml micrococcal nuclease). Cellular debris was removed by centrifugation at 10 000 xg for 5 min. Protein concentration was then determined by BioRad protein assay. 1/3 volume of 4X SDS sample loading buffer (200 mM Tris, pH 6.8, 400 mM dithiothreitol, 8% SDS, 0.2% bromophenol blue, 40% glycerol) was then added to the samples and then boiled for 5 min. Equal amounts of cellular protein (40-60 μg) were loaded per lane on 10% polyacrylamide-SDS gels. After electrophoresis, the proteins were transferred to nitrocellulose membrane in transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, 20% methanol) using the BioRad Trans-Blot Cell at a constant 0.25 A for 1 hr at 4°C. The membrane was then blocked with TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05 % Tween 20) supplemented with 3% non-fat dried milk for 1 h at room temperature. The membrane was then incubated with either monoclonal antibodies to cyclin B1 (Santa Cruz Biotech., GNS1) in TBST + 3% milk or cyclin A (Santa

Cruz Biotech., BF 683) in TBST + 1% milk at 1:200 dilution. For vimentin Western blots, vimentin monoclonal antibody (Boehringer Mannheim, clone V9) at the concentration of 0.3 μg/ml was incubated in TBST + 3% milk. The membrane was incubated for either 2 h at room temperature or overnight at 4°C. The membrane was then washed three times with TBST + 3% milk for 20 min each and then incubated for 1 h with HRP-conjugated anti-mouse antibody (Gibco) at 1:4000 in TBST + 3% milk. Finally, the membrane was washed 3-5 times and antigens were detected by enhanced chemiluminescence (Amersham).

2.4 [^{32}P]-Labelled Protein Extracts

BHK-21 cells were incubated with 50 μCi/ml [^{32}P]-orthophosphate in DMEM medium at the same time as the various drug incubations. The cells were collected and then lysed in 1/3 volume of 4 X SDS sample buffer. SDS polyacrylamide gel electrophoresis (SDS PAGE) was performed according to the method of Laemmli (1970). Equal amounts of protein extract were loaded onto each lane and the bands were visualized by autoradiography with Kodak X-OMAT film with intensifying screen at -70°C or quantified using a Molecular Dynamics Phosphorimager system.

2.5 Intermediate Filament Isolation

This protocol is similar to that of Steinert et al. (1982). Briefly, BHK-21 cells were collected, washed once with PBS and then lysed in isolation buffer (PBS, pH 7.4, 0.6 M KCl, 1% Triton X-100, 10 mM $MgCl_2$, 0.5 mM PMSF, 50 U/ml micrococcal nuclease). This procedure solubilized the cellular constituents, except for the intermediate filament caps, such as vimentin. The rest of the protocol was performed at 4°C to limit protease activity. The

intermediate filaments were then collected by centrifugation (3000 g for 5 min) and then washed three times with PBS with 5mM EDTA and 0.1 mM PMSF. The pellet was then solubilized in 1 X SDS sample buffer containing 8M urea and then run on a 8% SDS PAGE.

2.6 Immunoprecipitation of Vimentin

The immunoprecipitation procedure was very similar to that of Chou et al. (1989). Briefly, after drug incubation, the cells were collected and then lysed in 500 µl of lysis buffer (50 mM Tris pH 7.4, 5 mM EGTA, 5 mM EDTA, 10 mM sodium pyrophosphate, 0.2% SDS). The samples were boiled for 10 min and then treated with DNAse I (0.5 µg/ml). The rest of the protocol was performed at 4°C to limit protease degradation. Debris was removed by centrifugation 5 min at 10 000 xg. Then Triton X-100 was added to final concentration of 2%, and leupeptin and pepstatin to a final concentration of 2 µg/ml. The samples were precleared with 20 µl of 1:1 slurry of protein A-sepharose beads for 1 h and then 2.0 µg of vimentin antibody was added overnight. Then 20 µl of a 1:1 slurry of protein A-sepharose was incubated for 2 h on a nutator. The beads were collected by centrifugation (800xg) and washed three times with lysis buffer. The samples were analysed by SDS PAGE as described before.

2.7 Phosphorylation of Vimentin by PKC and PKA.

Vimentin and desmin were isolated from BHK cells as described in the vimentin isolation protocol. The intermediate filaments were further purified by solubilizing the proteins in disassembly buffer (0.1 M Tris, pH 7.4, 25 mM β-mercaptoethanol, 8 M urea) and then removing the urea by dialyzing against the buffer (5 mM Tris, pH 7.4, 1 mM dithiothreitol, 1 mM

EGTA, 0.17 M KCl). The significant proteins purified were vimentin and desmin as seen by a SDS gel stained with coomassie blue.

Vimentin was phosphorylated by the catalytic subunit of bovine heart cAMP-dependent kinase (Sigma). 5-10 μg of vimentin was phosphorylated with 0.5 μg kinase in PKA buffer (20mM Tris pH 7.4, 1 mM DTT, 0.1 M NaCl, 0.05 mM ATP, 12 mM $MgCl_2$, 2 μCi/μl [γ^{32}P]-ATP) for one hour at 30°C. Similar amounts of vimentin were phosphorylated with 5 ng of protein kinase C (Promega) in PKC buffer (20 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, 0.2 mg/ml phosphatidyl serine, 2 μCi/μl [γ^{32}P]-ATP) for one hour at 30°C. The reactions were stopped by the addition of 1/3 volume of 4 X SDS sample buffer.

2.8 Tryptic Peptide Mapping

2.8.1 Purification of vimentin by electrophoresis

Cells were pre-labelled with 50 μCi/ml [^{32}P]-orthophosphate in phosphate-free DMEM medium supplemented with dialysed FBS for 4-8 hours. Then drugs were added for two hours. The cells were collected and isolated using the vimentin isolation protocol as described before. *In vitro* labelled vimentin was phosphorylated as described before. Vimentin and desmin were separated on a preparative 8% polyacrylamide SDS gel. The gel was stained with coomassie blue, exposed wet to autoradiography and then the upper radioactive vimentin band was excised. The rest of the protocol for both tryptic mapping and phosphoamino analysis was similar to Boyle et al. (1991).

2.8.2 Elution of vimentin from gel slices

The gel slices and 500 µl of freshly prepared 50 mM ammonium bicarbonate were placed in 1.5 ml microfuge tubes and the gel slices were crushed with a stir rod. Ammonium bicarbonate was added to a final volume of 1 ml. To each tube, 50 µl of β-mercaptoethanol and 10 µl of 10% SDS were added. The samples were vortexed briefly, boiled for 3-5 min and then placed on a nutator at 37°C overnight to elute the protein. The tubes were then vortexed, centrifuged at 12 000 xg to remove the debris and the supernatant was carefully transferred to a new tube. A second volume (300-500 µl) of ammonium bicarbonate containing SDS and β-mercaptoethanol was added to wash the gel pieces and extract more protein. The samples were incubated at 37°C for another one hour, then the gel debris was removed by centrifugation and the supernatant was removed and combined with the first supernatant (≈ 1.2 ml). The tubes were then chilled on ice.

2.8.3. Concentration by TCA precipitation

To concentrate the labelled protein and remove SDS, the protein was precipitated in the presence of carrier protein (RNase) and 15-20% trichloroacetic acid (TCA). To each tube, 20 µg of RNase and 250 µl ice-chilled 100% TCA were added, and the tubes were vortexed and placed on ice for 2 hours. The TCA precipitate was collected by centrifugation in a microfuge for 10 min at 4°C at maximum speed. The supernatant was carefully removed and then the precipitate was washed with ice-cold 95% ethanol. The samples were vortexed briefly and centrifuged for 5 min. The supernatants were removed and the pellets were air-dried.

2.8.4. Oxidation and Trypsinization of Vimentin

The proteins were fully oxidized in the presence of performic acid. The amino acids methionine and cysteine had be oxidized to prevent the artefactual separation of oxidation isomers during chromatography. Performic acid was formed by addition of 900 µl of formic acid and 100 µl of hydrogen peroxide (33%) in a microfuge tube. The mixture was incubated at room temperature for 30-60 min and then chilled on ice.

The TCA precipitate was dissolved in 100 µl ice-cold performic acid and oxidized at 0°C for 60 min. 10 µl was removed for phosphoamino analysis and to the rest of the sample, 400 µl of water was added to dilute the performic acid and the samples were frozen in an ethanol-dry ice bath. The frozen samples were then lyophilized to completion (overnight).

The oxidized protein pellet was then resuspended in 50 µl ammonium bicarbonate solution, pH 8.0-8.3. The pellet was vortexed well and then spun down briefly. For standard trypinization, 10 µg of modifed, sequencing grade trypsin (Boehringer Mannheim) at the concentration of 10 mg/ml was added to each sample and incubated at 37 °C for 4-6 hours. Then a second aliquot of trypsin (10 µg) was added and incubated overnight for complete digestion of the protein.

The samples were then washed, lyophilized and dissolved repeatedly to remove any ammonium bicarbonate and insoluble protein. To the digests, 600 µl of water was added and then lyophilized to dryness using a Speed-Vac. Successive volumes of 400, 300 and 200 µl of water were added to the digests and repeatedly lyophilized. Then the samples were resuspended with 300 µl of pH 1.9 buffer (formic acid, acetic acid, water in the ratio of 1:3:36) and vortexed well. The samples were centrifuged for 5 min in a microfuge at room temperature to remove any particulate debris and the supernatant was

transferred to a new tube and relyophilized. The samples were then dissolved in 10 µl of pH 1.9 buffer and the amount of radioactivity was determined by Cerenkov counting.

2.8.5. First Dimension Electrophoresis

The first dimension of separation for the peptides is the electrophoresis of the peptides on thin layer chromatography (TLC) plates (Merc Pre-Coated TLC plates Cellulose). Each sample (800-1200 cpm) was loaded onto a TLC plate and the electrophoresis was done at 1000 V and 15 mA for one hour in pH 1.9 electrophoresis buffer. The plates were then dried in a fume hood. The apparatus used for the first dimension was the Hunter Thin Layer Peptide Mapping System.

2.8.6. Second Dimension Chromatography

The second dimension of separation is by thin layer chromatography. Phospho-chromatography buffer (n-butanol:pyridine:acetic acid:water 15:10:3:12) was added to chromatography tanks and left for 2-12 hours to equilibrate the liquid and the gas phases of the system. The plates were placed in a 6-plate holder and then quickly placed into the tank. The whole chromatography process was usually carried out for 9-12 hours until the solvent front reached about 3 cm from the top of the plates. The plates were then air dried in a fume hood and then exposed to autoradiography.

2.9. Phosphoamino analysis (PAA)

After performic acid treatment, 10 µl of the each sample was resuspended in 100 µl 6.0 N HCl, vortexed, then centrifuged briefly and incubated at 110 °C for one hour. The samples were then lyophilized in a

speed vac, resuspended in 5-10 µl of pH 1.9 buffer which contains 15 parts of buffer to 1 part of PAA standards (1.0 mg/ml each phosphoserine, phosphothreonine and phosphotyrosine) and loaded onto TLC plates. The first dimension electrophoresis was done in pH 1.9 buffer at 1.5 kV for 20 min and the second dimension in pH 3.5 buffer (acetic acid: pyridine:water, 10:1:189) at 1.3 kV for 16 min. The plates were dried in a fume hood and the phosphoamino acid standards were visualized by spraying the plates with 0.50 % ninhydrin in n-butanol. The plates were then put in a 65°C oven for at least 15 min to develop the stain and the radioactive spots were detected by autoradiography.

2.10. Immunofluorescence studies

BHK-21 cells were grown on poly-L-lysine coated coverslips overnight at a confluence of approximately 50-70%. The cells were treated with drugs and the coverslips were washed in PBS, fixed 10 min in 3.7% paraformaldehyde in PBS and then washed twice with KB solution (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.1% BSA). The vimentin primary monoclonal antibody (Boehringer Mannheim) was incubated with the coverslipsfor 30 min at room temperature in a humidified chamber (1:7 dilution) and then rinsed with KB twice for 10 min. The secondary antibody used was fluorescein isothiocyanate-conjugated goat anti-mouse IgG at 1:100 or Cy3-conjugated Affinpure goat anti-mouse IgG_1 (Jackson ImmunoResearch Lab) at 1:750. The coverslips were subsequently washed twice for 10 min with KB and then rinsed in PBS + bisbenzimide. The coverslips were mounted with fluormount (0.2 M n-propyl gallate in PBS and 90% glycerol). Cells were photographed on Kodak Tmax 400 film using a Zeiss Axiophot microscope.

2.11. Cytotoxicity Studies

HeLa cells were trypsinized and plated thinly on 60 mm petri dishes with grids. The cells were allowed to attach overnight at 37°C in 5% humified CO_2. The next day, drugs were added for 1-2 h and then the cells were washed gently twice with fresh medium. Every few days, the medium was replaced. After 7-10 days, the colonies were counted. A colony was defined as a group of cells greater than 10.

Chapter 3

Effects of Fostriecin on Intracellular Protein Phosphorylation

3.1 Fostriecin induces the hyperphosphorylation of a 55 kDa protein.

To investigate the effects of fostriecin on cellular protein phosphorylation, BHK-21 cells were incubated with fostriecin and [^{32}P]-orthophosphate for 2 h. The cells were lysed in SDS sample buffer, and the proteins were separated by SDS PAGE and analysed by autoradiography. Fostriecin did cause a slight general increase in protein phosphorylation over untreated cells (Fig. 6). However, a single protein band at 55 kDa became hyperphosphorylated (Fig. 6, lane 2). Incubation of cells with 1 μM okadaic acid, a known protein phosphatase inhibitor (Cohen et al., 1990), resulted in a very similar pattern, with the hyperphosphorylation of a single 55 kDa protein (Fig. 6, lane 4). The 55 kDa protein was not hyperphosphorylated in cells treated with ascorbic acid at the concentration present in the fostriecin preparation or with DMSO, the solvent for okadaic acid (Fig. 6, lanes 1, 3).

Phosphorylation of the 55 kDa protein was quantitated as a function of time after addition of fostriecin and [^{32}P] orthophosphate. It was detectable after 0.5 h and increased over the two-hour period (Fig. 7 A). When quantified as a function of fostriecin concentration, vimentin phosphorylation was detectable at 50 μM fostriecin and reached a maximum level at 200 μM (Fig. 7 B).

3.2 Identification of the 55 kDa protein as vimentin.

The protein phosphatase inhibitors okadaic acid and calyculin A have been reported to induce the hyperphosphorylation of vimentin, a 55 kDa

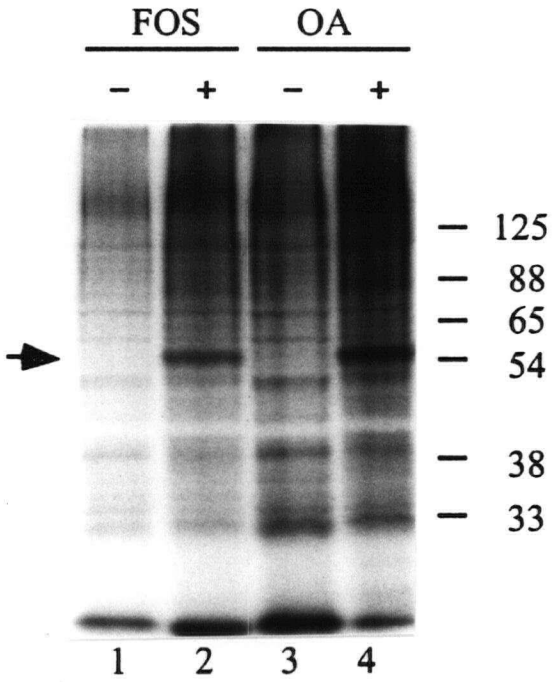


Figure 6. The hyperphosphorylation of a 55 kDa protein induced by fostriecin and okadaic acid. Cells were incubated with 50 μCi/ml [^{32}P]-orthophosphate and either with ascorbic acid (lane 1), 200 μM fostriecin (lane 2), DMSO (lane 3) or 0.5 μM okadaic acid (lane 4) for 2 h at 37°C. Cellular extracts were separated by SDS PAGE and [^{32}P] was visualized by autoradiography. The arrow indicates the position of the 55 kDa band.

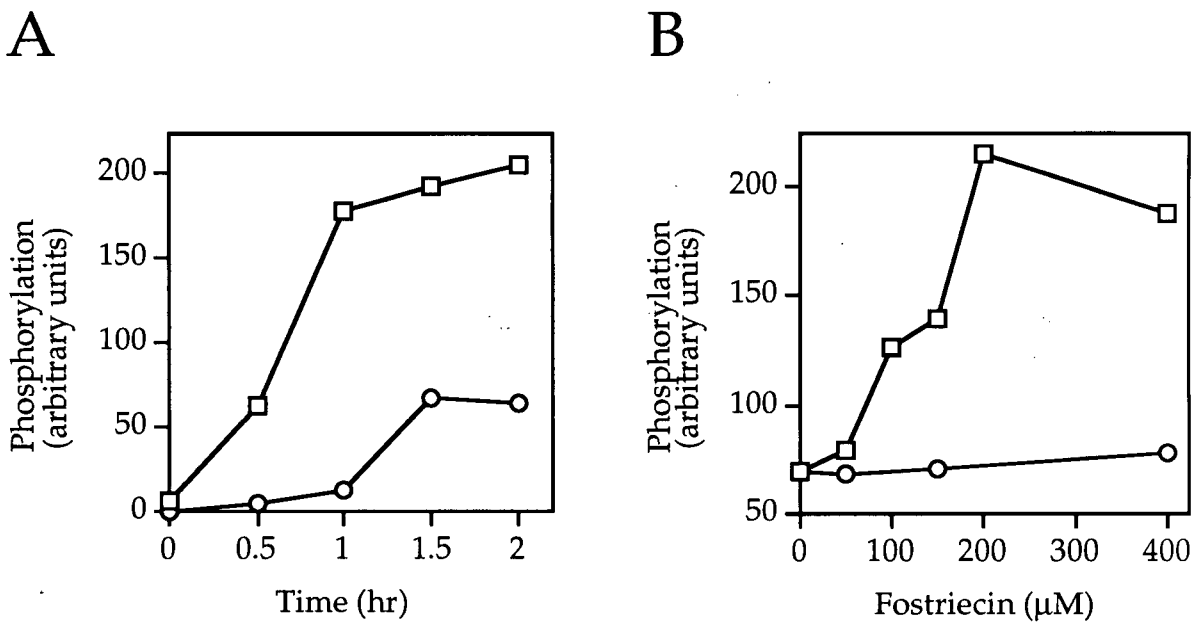


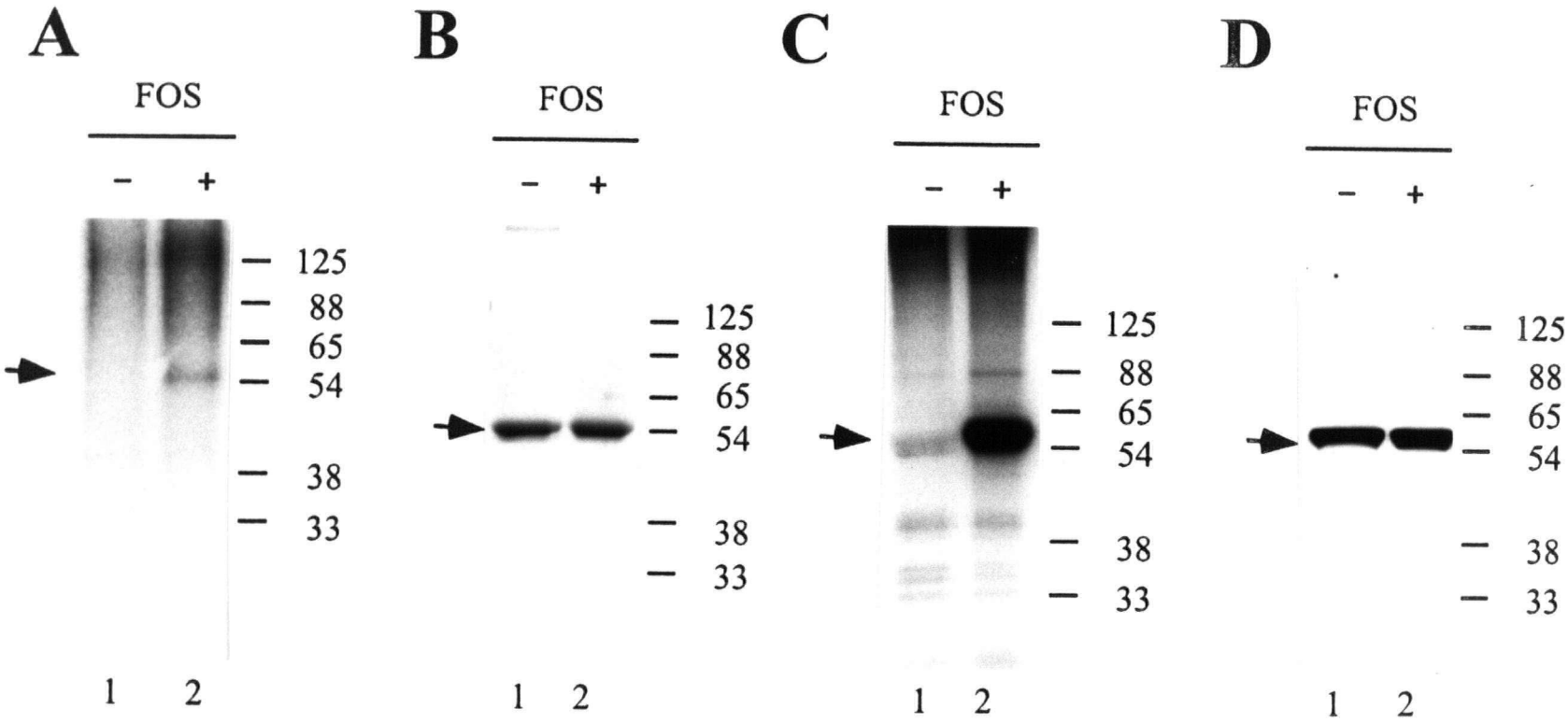
Figure 7. Hyperphosphorylation of the 55 kDa protein as a function of time and fostriecin concentration. Cells were incubated with 50 μCi/ml [^{32}P]-orthophosphate and either ascorbic acid (O) or 200 μM fostriecin (□) for the indicated times (A), or with 50 μCi/ml [^{32}P]-orthophosphate and the indicated concentrations of fostriecin (□) or corresponding ascorbic acid concentrations (O) for 2 h (B). Cellular extracts were separated by SDS PAGE and the phosphorylation of the 55 kDa band was quantitated using a Molecular Dynamics PhosphorImager. This figure represents one typical experiment.

protein (Chartier et al., 1991; Hirano and Hartshorne, 1993; Lee et al., 1992). To determine whether the 55 kDa protein hyperphosphorylated by fostriecin was vimentin, extracts were prepared from cells treated with [^{32}P]-orthophosphate and either ascorbic acid or fostriecin and subjected to immunoprecipitation with a monoclonal antibody to vimentin. As shown in Fig. 8 A, the antibody immunoprecipitated the hyperphosphorylated 55 kDa protein from fostriecin-treated cells (Fig. 8 A, lane 2), but not from ascorbic acid-treated cells (Fig. 8 A, lane 1). The [^{32}P]-labelled 55 kDa band was not detected when the vimentin antibody was omitted from immunoprecipitation reactions (not shown).

Intermediate filaments can be purified from BHK-21 cells on the basis of their insolubility using the procedure of Steinert et al. (1982). Intermediate filaments were purified from cells treated with [^{32}P]-orthophosphate and ascorbic acid or fostriecin and analysed by SDS PAGE and autoradiography. Purified preparations contained two closely migrating protein bands- vimentin and desmin- at about 55 kDa (Fig. 8 B). Autoradiography showed that the hyperphosphorylated 55 kDa band was highly enriched in intermediate filament preparations from cells treated with fostriecin compared with cells treated with ascorbic acid (Fig. 8 C, lanes 1 and 2). Furthermore, Western blot analysis of purified vimentin preparations showed strong reaction of vimentin antibodies with the 55 kDa protein (Fig. 8 D). Electrophoretic separation of desmin and vimentin was achieved using longer gels which showed that the phosphorylation of desmin was not stimulated by fostriecin treatment (not shown). Together these

Figure 8. The intermediate filament vimentin is hyperphosphorylated by fostriecin.

Cells were incubated with 50 μCi/ml [^{32}P]-orthophosphate and either ascorbic acid (lane 1) or 200 μM fostriecin (lane 2) for 2 h at 37°C. The intermediate filament vimentin was immunoprecipitated (A) or purifed (B, C) from [^{32}P]-labelled cellular extracts. The bands were visualized by autoradiography (A, C) or coomassie blue staining (B). Western blotting was carried out on the purified intermediate filament preparation (D) and detected by enhanced chemiluminescence. The arrow indicates the position of the vimentin protein.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B
FOS
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1 2
C
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- +
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88
65
54
38
33
1 2
D
FOS
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results demonstrate that the 55 kDa whose phosphorylation is stimulated by fostriecin is vimentin.

3.3 Fostriecin causes morphological changes in vimentin intermediate filaments.

Microscopic examination of cells treated with 100 μM fostriecin showed that cells began to round up afer 30 min. This was complete after 1 h, at which time most cells could be detached from the dish surface by shaking. This morphological change was not due to cell death because most of the rounded cells reattached when fostriecin was washed away. The morphological characteristics of the intermediate filament network were studied by indirect immunofluorescence using a monoclonal antibody to vimentin. Interphase control cells treated with ascorbic acid showed a characteristic network of vimentin filaments spanning the cytoplasm (Fig. 9 A). Mitotic control cells treated with ascorbic acid showed a diffuse staining indicative of vimentin depolymerization (Fig. 9 C). Cells treated with fostriecin had a different appearance. Those with interphase nuclei nevertheless showed a marked change in the organization of vimentin filaments, which became clustered around the nucleus (Fig. 9 B). Those in mitosis were similar to mitotic cells from control cultures.

3.4 Staurosporine-sensitive kinases phosphorylate vimentin exclusively at serine in fostriecin-treated cells.

As a first step in determining how fostriecin induces vimentin hyperphosphorylation, we identified the hyperphosphorylated amino acid residues. Vimentin was isolated from cells treated with [^{32}P]-orthophosphate and ascorbic acid or fostriecin and subjected to acid hydrolysis and

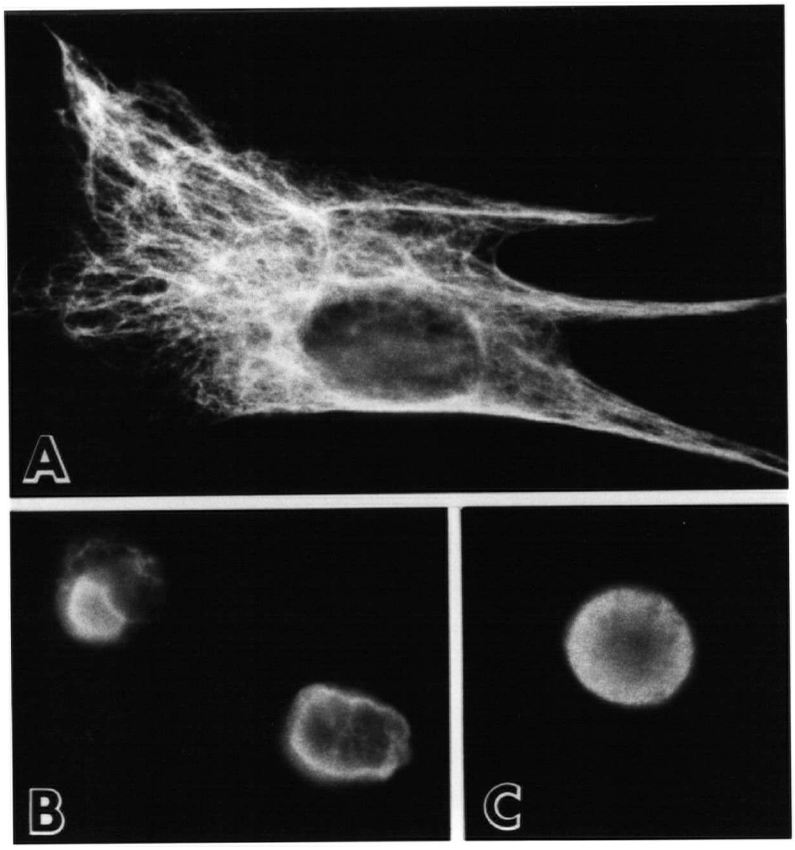


Figure 9. Morphological changes in the vimentin intermediate filament network induced by fostriecin. Cells were treated with ascorbic acid (A), 200 μM fostriecin (B) or 100 ng/ml nocodazole (C) for 2 h. The cells were harvested, reacted with monoclonal vimentin antibody and CY3-conjugated goat anti-mouse IgG and then visualized by fluorescence microscopy.

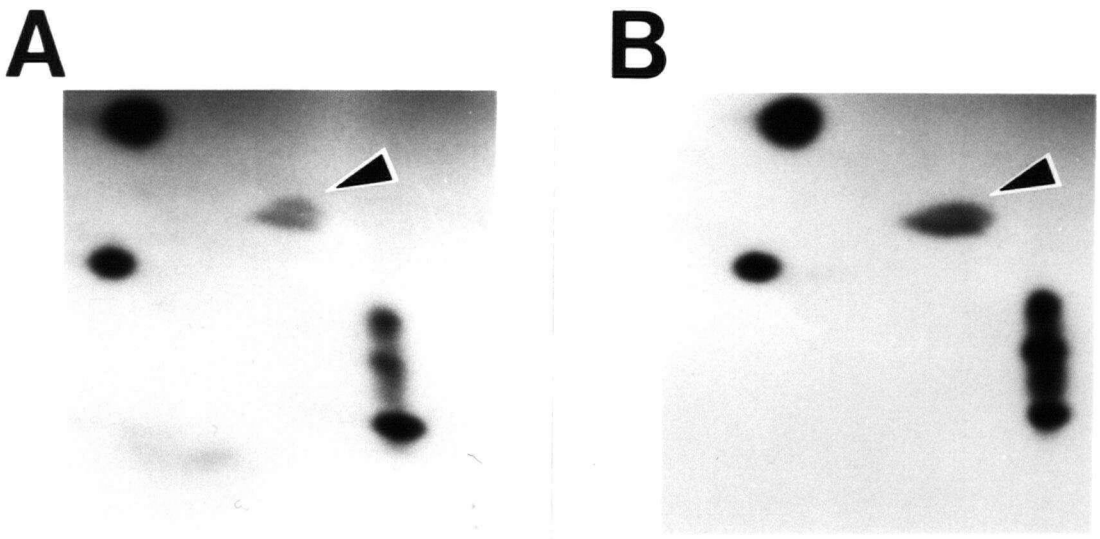
phosphoamino analysis. Fig. 10 shows that vimentin was phosphorylated exclusively at serine residues in controls (Fig. 10 A) and in fostriecin-treated cells (Fig. 10 B). No phosphorylated tyrosine or threonine residues were detected. The inorganic [^{32}P] is seen on the upper left corner and the partial hydrolyzed products are at the lower right corner (Fig. 10).

Next we examined the sensitivity of vimentin phosphorylation to protein kinase inhibitors. The potent Ser/Thr and weak Tyr kinase inhibitor staurosporine (200 nM) added at the same time as 200 μM fostriecin and 50 μCi/ml [^{32}P]-orthophosphate inhibited vimentin hyperphosphorylation by at least 70% (Fig. 11, lane 4).

To investigate the role of cdc2 in fostriecin-induced vimentin phosphorylation, we used a genetic approach and the inhibitor olomucin. The mouse FT210 cell line has a temperature-sensitive *cdc2* allele. These cells grow normally at 32°C but at 39°C they lose their cdc2 and are unable to enter mitosis (Th'ng et al., 1990). Fostriecin's ability to stimulate vimentin phosphorylation was not diminished in FT210 cells at 39°C, indicating that cdc2 was not involved (Fig. 12, lane 4). The cdc2 inhibitor olomucin (50 μM) showed less than 15% inhibition (Fig. 13). These results suggest that the staurosporine-sensitive kinase responsible for over 70% of the fostriecin-induced vimentin hyperphosphoryation is not cdc2. To investigate the role of PKC, we used the PKC inhibitors calphostin C (1 μM), UCN-01 (300 nM) and GF109203X (300 nM). All three showed about 50% inhibition (Fig. 13). The S6 kinase inhibitor rapamycin and the calmodulin-dependent kinase II inhibitor KN-93 showed no inhibition.

Taken together, these results show that a staurosporine-sensitive Ser kinase is responsible for over 70% of the fostriecin-induced vimentin hyperphosphorylation. This kinase is unlikely to be cdc2 but could be PKC.

Figure 10. Vimentin phosphorylation induced by ascorbic acid or fostriecin occurs only on serine residues. Cells were treated with 50 μCi/ml [^{32}P]-orthophosphate and ascorbic acid (A) or 200 μM fostriecin (B). The intermediate filaments were isolated and separated by SDS PAGE. The vimentin band was excised, eluted and then subjected to HCl hydrolysis. The resulting amino acids were separated by electrophoresis at 1500 V for 20 min in pH 1.9 buffer (formic acid:acetic acid:water, 1:3:36) in the first dimension and at 1300 V for 16 min in pH 3.5 buffer (acetic acid: pyridine:water, 10:1:189) in the second dimension. The cold phosphoamino acid standards were visualized by staining with ninhydrin and heating at 60°C for 15 min. The phosphoamino acids were visualized by autoradiography. The arrow indicates the position of the phosphoserine residues.


A
B

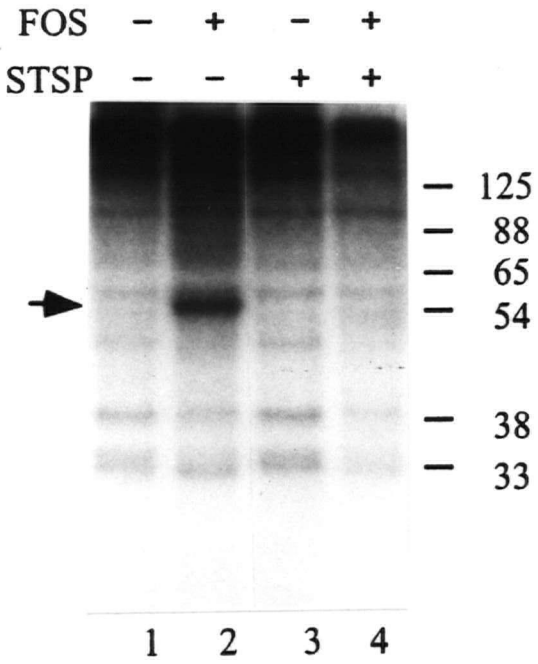


Figure 11. Fostriecin cannot induce vimentin phosphorylation in the presence of staurosporine. Cells were treated with 50 μCi/ml [^{32}P]-orthophosphate and either ascorbic acid (lanes 1, 3) or 200 μM fostriecin (lanes 2, 4) in the absence (lanes 1, 2) or presence of 200 nM staurosporine (lanes 3, 4). Cellular extracts were separated by SDS PAGE and [^{32}P] was visualized by autoradiography. The arrow indicates the position of the vimentin protein.

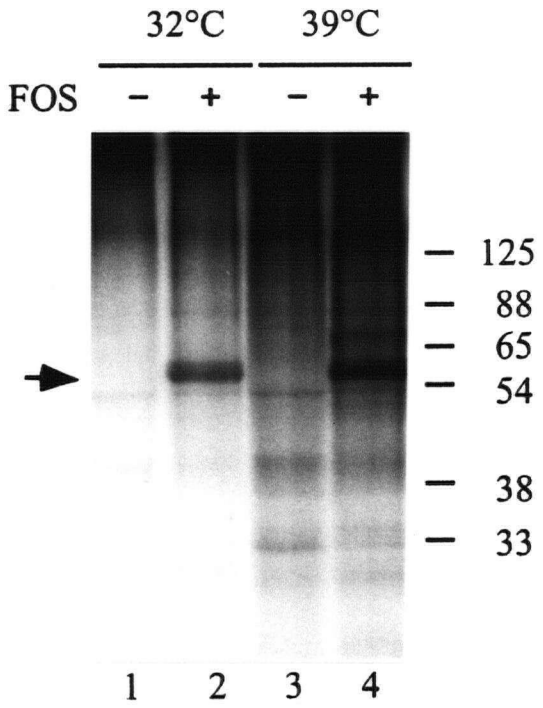


Figure 12. Vimentin phosphorylation occurs in the absence of cdc2 kinase activity. FT210 cells were either maintained at the permissive temperature of 32°C (lanes 1, 2) or arrested in G2 at the non-permissive temperature of 39°C (lane 3, 4). The cells were then treated with 50 μCi/ml [^{32}P]-orthophosphate and either ascorbic acid (lanes 1, 3) or 100 μM fostriecin (2, 4) for 2 h. Cells were collected, vimentin was isolated, separated by SDS PAGE and phosphorylated proteins were visualized by autoradiography. The arrow indicates the position of the vimentin protein.

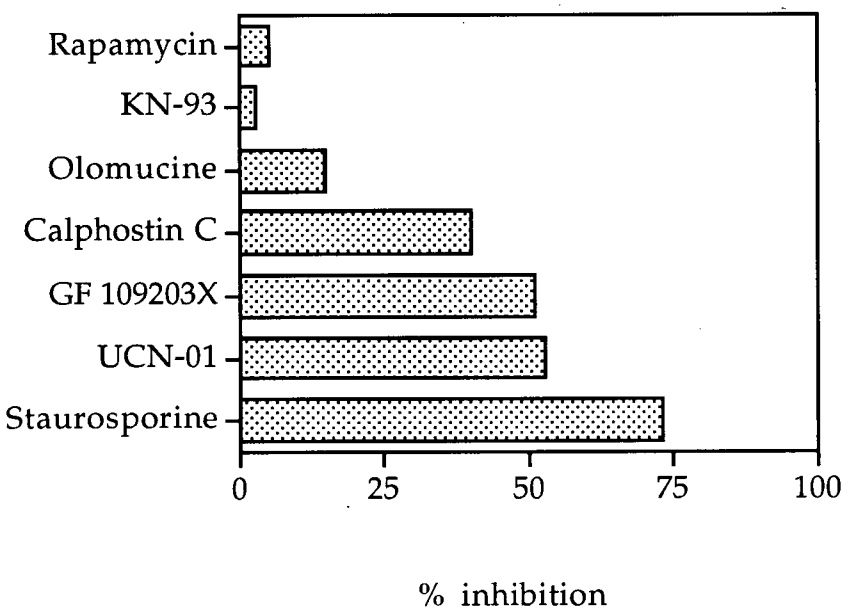


Figure 13. The inhibition of vimentin phosphorylation by protein kinase inhibitors. BHK-21 cells were preincubated with the various protein kinase inhibitors and 50 μCi/ml [^{32}P]-orthophosphate for 0.5 h. Then ascorbic acid or 200 μM fostriecin was added for 2 h at 37°C. Cellular extracts were separated by SDS PAGE and quantitation was carried out using a Molecular Dynamics PhosphorImager. The protein kinase inhibitors did not affect vimentin phosphorylation in cells treated with ascorbic acid (not shown). This figure represents one typical experiment.

3.5 Tryptic peptide mapping of phosphorylated vimentin.

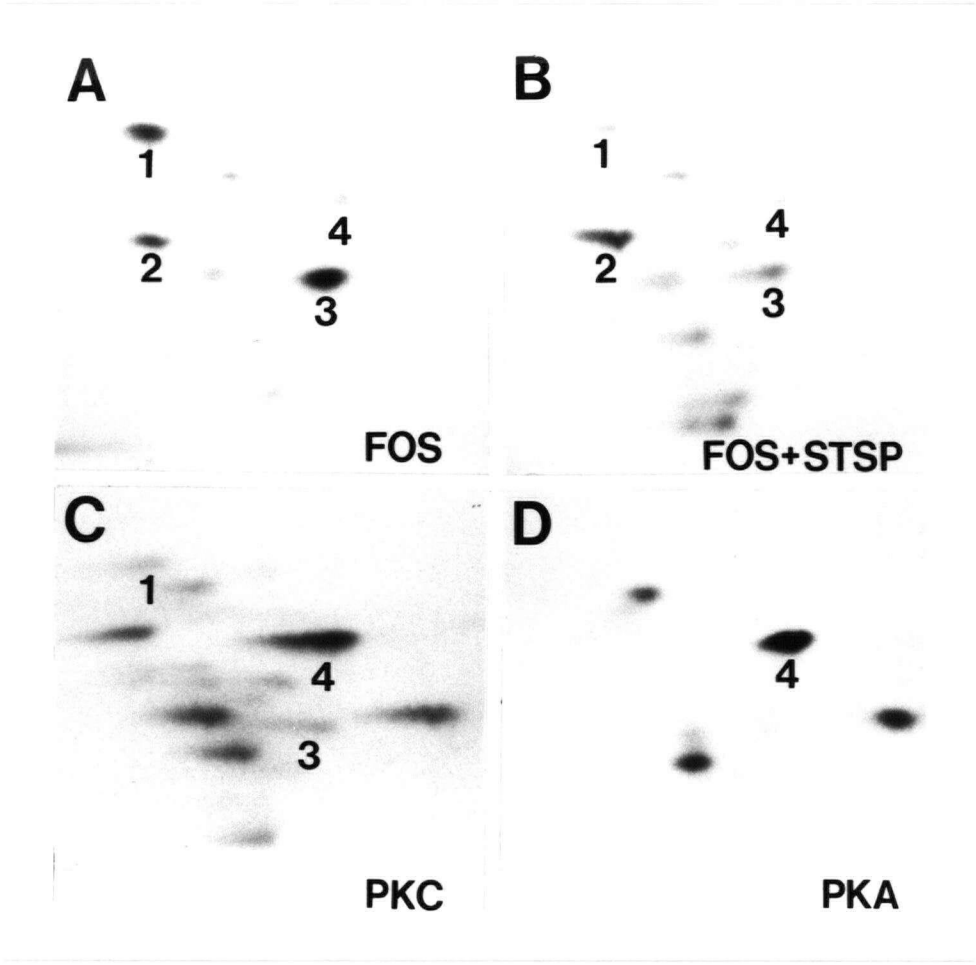
We further characterized vimentin phosphorylation in cells treated with fostriecin or with fostriecin and staurosporine by two-dimensional tryptic mapping. Fig. 14 A shows 3 major tryptic phosphopeptides (labeled 1-3) and 5 minor ones in vimentin isolated from cells treated with fostriecin alone. Fig. 14 B shows that the number of labeled phosphopeptides was unchanged by staurosporine treatment but that labeling of phosphopeptides 1 and 3 was significantly reduced by 60% and 65% respectively. This indicates that staurosporine-sensitive kinase(s) cause most of the fostriecin-induced vimentin hyperphosphorylation by increasing phosphorylation at two sites.

To determine the identity of the staurosporine-sensitive kinase(s) involved, we compared the phosphopeptide map of phosphorylated vimentin isolated from fostriecin-treated cells with that of vimentin phosphorylated by purified protein kinases *in vitro*. Vimentin is efficiently phosphorylated by PKC and PKA *in vitro* and these two kinases are thought to participate in interphase phosphorylation of vimentin (Ando et al., 1989; Chou et al., 1989; Geisler et al., 1989; Inagaki et al., 1987). At mitosis, vimentin is further phosphorylated by cdc2 (Chou et al., 1990; Chou et al., 1991). We note that staurosporine at the concentration of 200 nM used here would be expected to inhibit efficiently intracellular PKC (IC_{50}= 9nM) and cdc2 (IC_{50}= 7nM), but not PKA (IC_{50}= 1100 nM). Tryptic maps of vimentin phosphorylated by PKA or PKC *in vitro* were generated. PKC phosphorylated eight sites (Fig. 14 C). Phosphopeptides 1, 3 and 4 were also found *in vivo* whereas the other five sites were not found *in vivo*. PKA phosphorylated vimentin at four sites (Fig. 14 D). Phosphopeptide 4 corresponds to the minor *in vivo* site whereas the others do not. Thus, PKC phosphorylated the two sites that are staurosporine-sensitive and highly phosphorylated in fostriecin-

treated cells. PKA did not phosphorylate any of these major *in vivo* sites. Additionally PKC and PKA both phosphorylated site 4, a minor *in vivo* site.

Taken together, these results show that fostriecin stimulates the phosphorylation of serine residues in vimentin at three sites which are normally phosphorylated in interphase but to a lesser degree. Two of these sites are sensitive to staurosporine *in vivo* and can be phosphorylated by PKC *in vitro*. Since staurosporine is a potent inhibitor of PKC, this suggests that PKC may be one of the kinases that is responsible for the increase in vimentin phosphorylation during incubation of cells with fostriecin.

Figure 14. Three tryptic peptides detected following fostriecin-induced hyperphosphorylation of vimentin. [^{32}P]-labelled vimentin was isolated from cells treated with fostriecin and without (A) or with staurosporine (B). Vimentin was also phosphorylated *in vitro* using purified PKC (C) and PKA (D) and γ[^{32}P]-ATP. The samples were digested with trypsin and 800-1200 cpm were loaded onto TLC plates and separated in the first dimension by electrophoresis at 1000 V and 15 mA for 1 h in pH 1.9 electrophoresis buffer (formic acid:acetic acid:water, 1:3:36), and in the second dimension by chromatography in chromatography buffer (n-butanol:pyridine:acetic acid:water 15:10:3:12) for 9-12 h. The tryptic phosphopeptides were visualized by autoradiography.


A
1
2
4
3
FOS
B
1
2
4
3
FOS+STSP
C
1
4
3
PKC
D
4
PKA

Chapter 4

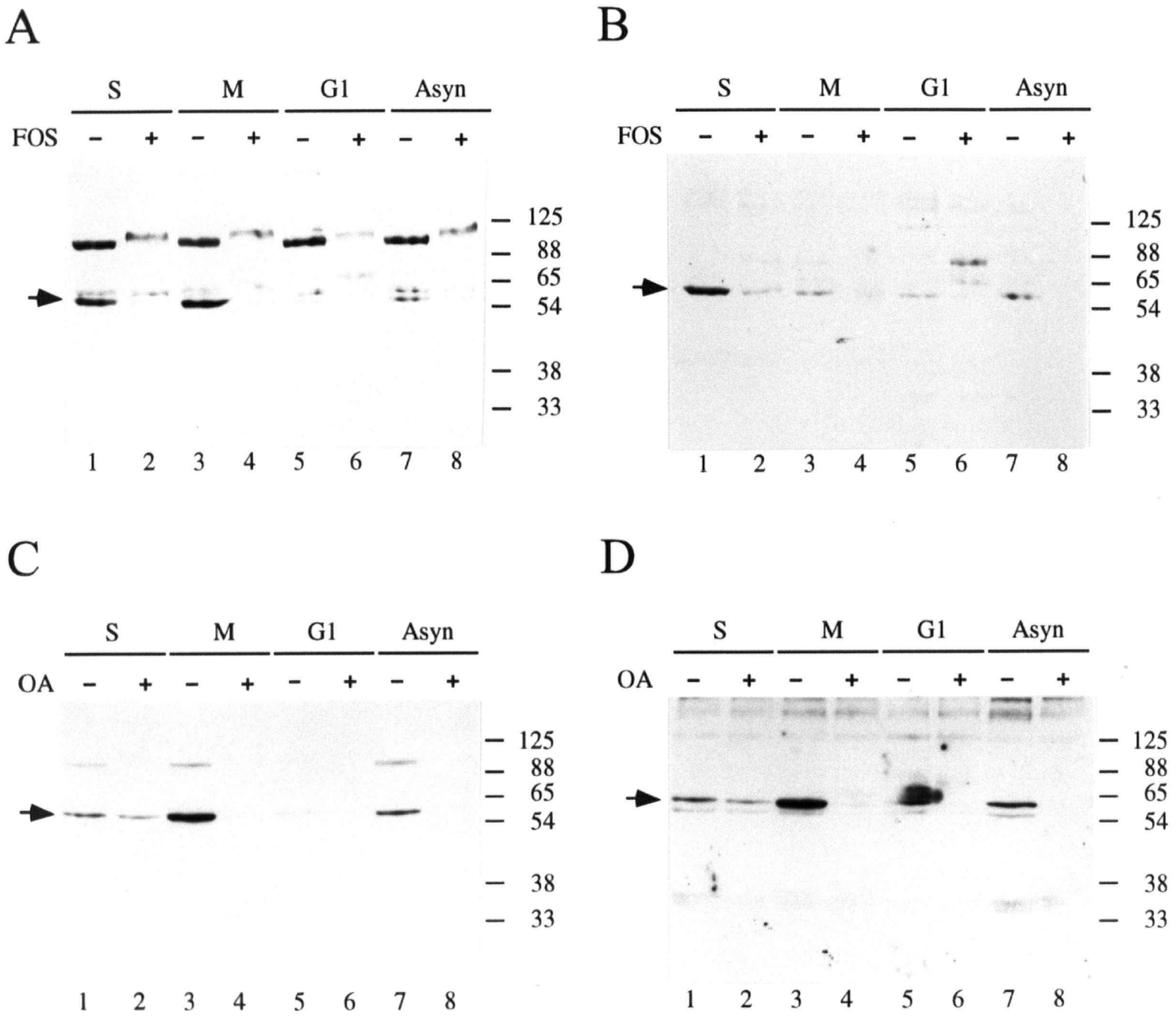
Fostriecin Induces Premature Cyclin Degradation

4.1 Protein phosphatase inhibitors uncouple the degradation of cyclin A and cyclin B from mitosis.

As documented in Guo et al. (1995), when tsFT210 cells were blocked in late G2 phase without cdc2 activity, treatment with fostriecin caused chromosome condensation in a large number of cells, as well as degradation of cyclins A and B. Similarly, fostriecin induced cyclin A and cyclin B degradation in tsFT210 cells arrested in mitosis with active cdc2 using the microtubule-depolymerizing agent nocodazole. We wanted to determine whether cyclin degradation induced by fostriecin required mitosis or could also happen in interphase.

Human epitheloid carcinoma HeLa cells were arrested in G0/G1, S phase or mitosis or were left asynchronous (Fig. 15). The cells were then incubated with either 200 μM fostriecin or its vehicle ascorbic acid, or with 1 μM okadaic acid or its vehicle DMSO for 2 h and cellular proteins were separated by SDS PAGE and immunoblotted with monoclonal antibodies to cyclin A (Fig. 15 B, D) or to cyclin B (Fig. 15 A, C). In keeping with its known pattern of accumulation (Girard et al., 1995), the level of cyclin B was very low in G1 phase, higher in S phase and maximal in cells arrested in mitosis, with intermediate levels in asynchronous cells. Cyclin A also increases in amount during S and G2, but unlike cyclin B, is unstable in cells arrested in mitosis with microtubule inhibitors, such as nocadozole (Dorée and Galas, 1994). This was also seen here in Fig. 15 B, lanes 5 and 6. Upon incubation with fostriecin or okadaic acid, cyclin A and cyclin B levels decreased dramatically

Figure 15. Fostriecin induces cyclin degradation at various stages of the cell cycle. HeLa cells were synchronized to G1, S and M as described in the Materials and Methods (section 2.1). Ascorbic acid, 200 μM fostriecin (A, B) or 1 μM okadaic acid (C, D) was added for 2 h at 37°C. The cells were collected and cellular extracts were loaded on a 10% SDS PAGE and immunoblotted with cyclin A (B, D) or cyclin B (A, C) antibodies. The Western blots were detected by ECL (Amersham). The position of the cyclin band is indicated by the arrows.


A
S
M
G1
Asyn
FOS
– + – + – + – +
125
88
65
54
38
33
1 2 3 4 5 6 7 8
B
S
M
G1
Asyn
FOS
– + – + – + – +
125
88
65
54
38
33
1 2 3 4 5 6 7 8
C
S
M
G1
Asyn
OA
– + – + – + – +
125
88
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38
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1 2 3 4 5 6 7 8
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G1
Asyn
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– + – + – + – +
125
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1 2 3 4 5 6 7 8

from all cell cycle stages (Fig. 15). Degradation of both cyclin A and cyclin B was detectable 1 h after addition of fostriecin, and was 95% complete after 2 h (Fig. 16 A). In contrast, the levels of cyclins A and B remained stable in cells incubated with ascorbic acid or DMSO. Fig. 16 B shows the concentration dependence of this effect during 2 h of incubation. Degradation of cyclin A and cyclin B was detectable at 100 μM and over 80% of the cyclins were degraded at 150 μM (Fig. 16 B). Different concentrations of ascorbic acid did not affect cyclin levels. Coomassie Blue staining of the gels revealed no general protein degradation (not shown). Similar results were obtained with human lymphoma Jurkat cells (data not shown). These results establish that fostriecin can stimulate cyclin degradation in human cancer cell lines. Since cyclin A and cyclin B are normally very stable during interphase, the induction of cyclin degradation suggested either that all cells were undergoing mitosis or that fostriecin activated premature cyclin degradation in interphase cells. However, fostriecin induced entry into mitosis in less than 10% of asynchronous HeLa cells as detected by mitotic spreads under the conditions where it induced complete cyclin degradation (not shown). This suggests that cyclin degradation was not a consequence of cells undergoing mitosis, but was induced prematurely in interphase cells.

4.2 Cyclin degradation induced by fostriecin requires staurosporine-sensitive kinase(s).

We characterized the mechanism by which fostriecin induces cyclin degradation in more detail. The data in Chapter 3 have already shown that staurosporine inhibits fostriecin-induced protein phosphorylation (Fig. 11). To determine whether fostriecin triggers cyclin degradation by modulating protein phosphorylation, HeLa cells were preincubated with or without

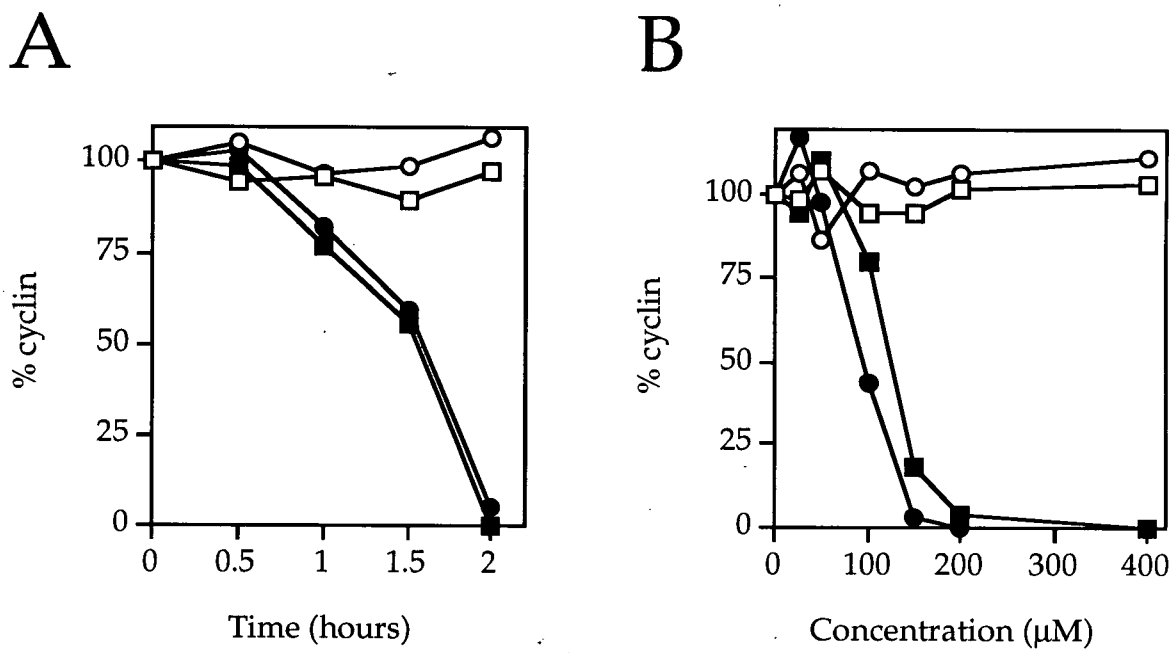


Figure 16. The premature degradation of cyclin A and cyclin B as a function of time and fostriecin concentration. HeLa cells were synchronized to S phase and released for 2 h before drug addition. In panel A, ascorbic acid (□, ○) or 200 μM fostriecin (■, ●) was added at time 0 and the cells were collected at various times. In panel B, various concentrations of ascorbic acid (□, ○) or fostriecin (■, ●) were added for 2 h. Cellular extracts were made and separated on 10% SDS PAGE gels. Western blot analysis was done with either cyclin A (■, □) or cyclin B (●, ○) monoclonal antibodies. The levels of cyclins were quantified by laser densiometry. The figure represents the average of two separate experiments.

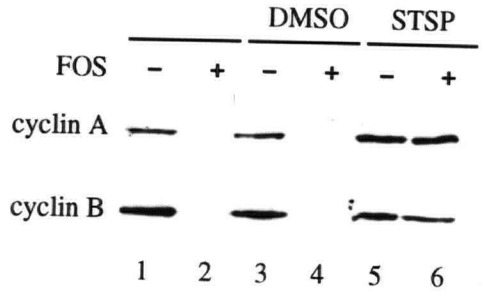
staurosporine for 0.5 h and ascorbic acid or 200 μM fostriecin was added for another 2 h. Immunoblotting of cell extracts showed that staurosporine inhibits the degradation of both cyclin A and cyclin B (Fig. 17 A). Similar results were also obtained with Jurkat cells (not shown). The protein kinase C inhibitors H-7 and calphostin C, the calmodulin antagonist W-7, the protein synthesis inhibitor cycloheximide and the phorbol ester 4β-phorbol 12-myristate 13-acetate did not prevent cyclin degradation (not shown). Our group showed previously that fostriecin induces cyclin degradation in FT210 cells depleted of cdc2 activity by incubation at the non-permissive temperature (Guo et al., 1995). Thus, cyclin degradation induced by fostriecin requires the activity of a staurosporine-sensitive protein kinase that is unlikely to be protein kinase C, a calmodulin-dependent kinase, or cdc2.

4.3 Proteasome inhibitors prevent fostriecin-induced cyclin degradation.

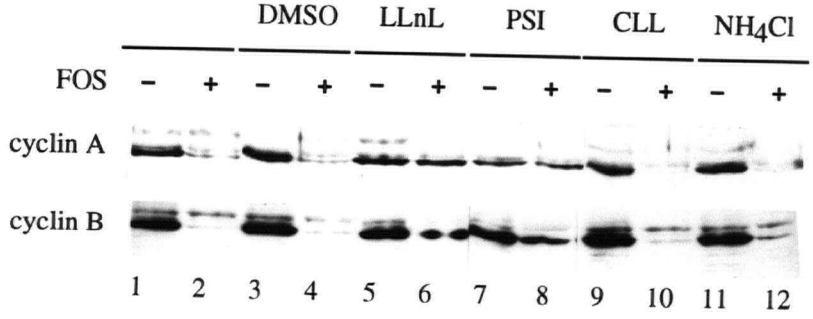
The cell permeable peptide aldehydes N-acetyl-Leu-Leu-norleucinal (LLnL) and Cbz-Ile-Glu(O-t-Bu)-Ala-leucinal (PSI) are potent *in vivo* inhibitors of the chymotrypsin-like activity of the proteasome (Figueiredo-Pereira et al., 1994; Pagano et al., 1995; Palombgella et al., 1994; Rock et al., 1994; Traenckner et al., 1994), the multisubunit complex responsible for cyclin degradation at mitosis (Ghislain et al., 1993). LLnL has been shown to inhibit the degradation of cyclin A and cyclin B at mitosis in CHO cells (Sherwood et al., 1993). HeLa cells were incubated with low concentrations of LLnL or PSI (5 μM) for 0.5 h before the addition of fostriecin or ascorbic acid for another 2 h. Immunoblotting showed that these inhibitors prevented fostriecin-induced degradation of cyclin A and cyclin B (Fig. 17 B). LLnL and PSI are potent *in vitro* inhibitors of neutral cysteine proteases and thus also inhibit calpains and lysosomal cathepsins in addition to the proteasome (Figueiredo-Pereira et

Figure 17. Fostriecin-induced cyclin degradation requires staurosporine-sensitive kinases and proteasome activity. HeLa cells were synchronized to S phase as described in Materials and Methods (section 2.1). They were then preinucated for 0.5 h with 200 nM staurosporine (A), 5 μM LnLL, 5 μM PSI, 25 μM CLL or 10 mM NH_4Cl (B). Then ascorbic acid or 200 μM fostriecin was added for 2 h at 37°C. The cells were collected and cellular extracts were separated by 10% SDS PAGE and immunoblotted with cyclin A or cyclin B antibodies. The Western blots were detected by ECL (Amersham).

A


DMSO
STSP
FOS
- + - + - +
cyclin A
cyclin B
1 2 3 4 5 6

B


DMSO
LLnL
PSI
CLL
NH4Cl
FOS
- + - + - + - + - + - +
cyclin A
cyclin B
1 2 3 4 5 6 7 8 9 10 11 12

al., 1994; Sasaki et al., 1990). Cbz-Leu-leucinal (CLL) is a strong calpain inhibitor but a weak proteasome inhibitor which can be used to inhibit calpain selectively in living cells (Figueiredo-Pereira et al., 1994). High concentrations of CLL (25 μM) did not inhibit fostriecin-induced cyclin degradation (Fig. 17 B). NH_4Cl neutralizes lysosomal acidity, causing a selective and complete inhibition of lysosomal proteases, including the cathepsins (Figueiredo-Pereira et al., 1994; Seglen, 1983). NH_4Cl at high concentration (10 mM) did not inhibit fostriecin-induced cyclin degradation (Fig. 17 B). This is strong evidence that the protease responsible for fostriecin-induced cyclin degradation is the proteasome and not a lysosomal enzyme or calpain.

4.4 Fostriecin cytotoxicity requires neutral cysteine protease activity

Since normal cell cycle progression requires cyclins, premature activation of the proteasome-dependent cyclin degradation pathway would be expected to inhibit cell proliferation strongly. If this is the case, then inhibiting proteasome activity during fostriecin treatment might offer protection against fostriecin cytotoxicity. Such experiments are difficult because protease inhibitors are generally themselves very toxic. However, the proteasome inhibitor LLnL at a concentration sufficient to prevent cyclin degradation (5 μM) shows no toxicity towards CHO cells during 1-2 h incubation (Sherwood et al., 1993). In three separate experiments, HeLa cells were incubated with or without protease inhibitors for 0.5 h before the addition of 200 μM fostriecin or ascorbic acid for 2 h. The drugs were then washed away by rinsing the cell monolayers with fresh culture medium and the cells allowed to form colonies for up to 10 days. We wanted to determine whether protease inhibitors would partially inhibit the cytotoxicity of

incubated HeLa cells with 200 μM fostriecin for 2 h. At this concentration, fostriecin induces complete cyclin degradation (Fig. 16B) and shows 100% cytotoxicity. The protease inhibitors did not inhibit the cytotoxicity of fostriecin (not shown).

We determined whether at a lower concentration of fostriecin, protease inhibitors could prevent the cytotoxicity. 100 μM fostriecin for one hour leads to 40-80% cell death (Table 4). Although the cytotoxicity of 100 μM fostriecin varied amongst the three trials, 5 μM LLnL partially inhibited fostriecin cytotoxicity in all experiments by 10-55% (Table 4). The other proteasome inhibitor PSI showed significant cytotoxicity on its own and could not be used to address this question (not shown). Interestingly, 5 μM CLL and 2 mM NH_4Cl also significantly reduced fostriecin cytotoxicity. However, fostriecin after 1 h at 100 μM does not induce detectable cyclin degradation (not shown). This result shows that neutral cysteine proteases inhibited by 5 μM LnLL, 5 μM CLL and 2 mM NH_4Cl are at least partially required for fostriecin cytotoxicity. This further indicates that degradation of proteins other than cyclins A and B must also play important roles in fostriecin's cytotoxicity.

Table 4- The cytotoxicity of fostriecin is prevented by inbibitors of neutral cysteine proteases.

Fostriecin 100 μM	% survival		
	Experiment		
	1	2	3
- [a]	16	59(1)[b]	21(3)
LnLL 5 μM	70	90(4)	30(1)
CLL 5 μM	50	85(4)	n/d [c]
NH_4Cl 2mM	59	91(3)	n/d

[a] The cells were preincubated with LnLL, CLL or NH_4Cl for 0.5 h and then ascorbic acid or 100 μM fostriecin was added for 1 h. The drugs were washed away and the cells were allowed for grow for 5-10 days.

[b] Mean and standard deviation (in parenthesis) of duplicate samples.

[c] Not determined.

Chapter 5

Discussion

5.1 A protein phosphatase is the likely *in vivo* target of fostriecin.

The results presented in Chapter 3 show that treatment of BHK-21 cells with fostriecin causes a large increase in the phosphorylation of a single 55 kDa protein, and that this protein is the intermediate filament protein vimentin. Intermediate filaments (IF) are one of three major cytoskeletal components found within many eukaryotic cells. Vimentin is found in mesenchymal cell types and in many transformed cell lines (Fuchs and Weber, 1994). Vimentin and other IFs were found to undergo changes in structural organization during cellular processes such as mitosis and differentiation (Chou et al., 1989). These changes in structural organization are modulated by protein phosphorylation as shown by *in vitro* studies with purified protein kinases (Geisler and Weber, 1988; Inagaki et al., 1988; Inagaki et al., 1987). *In vivo*, phosphorylation of vimentin is site-specific and is restricted to the 9 kDa N-terminal head domain (Ando et al., 1989; Geisler et al., 1989; Inagaki et al., 1987). During interphase, vimentin exists in a polymerized state visible as a filamentous network which spans the cytoplasm and provides tensile strength to the cells It is phosphorylated to a low degree at several sites (Fuchs and Weber, 1994). At mitosis, vimentin becomes hyperphosphorylated, causing the filaments to depolymerize and form cytoplasmic aggregates (Chou et al., 1990; Chou et al., 1991; Chou et al., 1989). The protein phosphatase inhibitors okadaic acid and calyculin A cause hyperphosphorylation of vimentin (Eriksson et al., 1992), disassembly and clustering of vimentin filaments around the nucleus (Lee et al., 1992) or in

patches in the cytoplasm (Chartier et al., 1991) and rounding up of the cells (Lee et al., 1992). Geisler et al. (1989) have also reported that phosphorylation of vimentin filaments *in vitro* can also be associated with filament bundling without extensive depolymerization. Our finding that fostriecin also causes these changes provides strong evidence that fostriecin acts *in vivo* as an inhibitor of protein phosphatases 1 and/or 2A, even though it bears no structural resemblance to okadaic acid or to calyculin A.

The lowest fostriecin concentration at which we observed stimulation of vimentin phosphorylation in 2 h is 50 μM (Fig. 2B), well above the IC_{50} for PP1 and PP2A *in vitro*. The drug concentration required to inhibit an enzyme in the cell depends on several factors including the intracellular concentration of the enzyme, the stability of the drug in the incubation medium, and the permeability of the plasma membrane to the drug (Cohen et al., 1990). The intracellular concentration of PP1 and PP2A is usually in the 0.1-1 μM range (Cohen et al., 1990). Inhibition would therefore be expected to require μM concentrations of fostriecin in the cell. Furthermore, fostriecin is particularly sensitive to oxidation; it loses 50% of its activity within 30 min in the tissue culture medium (Roberge et al., 1994). The permeability of the cell membrane to fostriecin is not well known. Therefore, our observation that fostriecin stimulates vimentin hyperphosphorylation when initially present in the culture medium at 50 μM and above is compatible with the notion that fostriecin inhibits vimentin phosphatases directly, and that the disruption of the kinase-phosphatase equilibrium causes hyperphosphorylation of vimentin and reorganization of intermediate filaments.

It has been shown that fostriecin can cause cells to enter mitosis prematurely, even when cells are normally prevented from doing so because their DNA is incompletely replicated or damaged (Roberge et al., 1994). Is the

hyperphosphorylation of vimentin caused by fostriecin similar to that which occurs normally at mitosis? Cdc2 is the mitotic kinase that phosphorylates vimentin during mitosis and mediates structural rearrangements (Chou et al., 1990; Chou et al., 1989) It specifically phosphorylates Ser-55 in the N-terminal non-α-helical domain (Chou et al., 1991). The data show that fostriecin can induce vimentin hyperphosphorylation even in cells that lack cdc2. The kinase(s) responsible for this hyperphosphorylation are probably interphase vimentin kinases rather than cdc2. Furthermore, another study has found that calyculin A stimulated the phosphorylation on the interphase sites and not on the mitotic sites of vimentin (Eriksson et al., 1992).

Vimentin phosphorylation by kinases active in interphase has been extensively studied (Ando et al., 1989; Geisler et al., 1989; Geisler and Weber, 1988; Inagaki et al., 1988; Inagaki et al., 1987). PKC and PKA have been identified as kinases that phosphorylate vimentin efficiently *in vitro* and are good candidates for interphase vimentin kinases (Lee et al., 1992). Specifically, PKC phosphorylated Ser-8, 9, 20, 25, 33 and 41, while PKA preferentially phosphorylated Ser-46 in *in vitro* studies (Ando et al., 1989). Furthermore, both kinases phosphorylated Ser-6, 24, 38, 50 and 65 (Ando et al., 1989). Therefore, we determined whether the sites that became hyperphosphorylated during fostriecin treatment were PKC or PKA sites by comparing tryptic phosphopeptide maps of vimentin phosphorylated *in vivo* in the presence of fostriecin with maps of vimentin phosphorylated *in vitro* by PKC or PKA. Two of the three major tryptic phosphopeptides obtained after fostriecin treatment were also phosphorylated by PKC *in vitro*, as shown by comigration. However, these sites were relatively weakly phosphorylated by PKC *in vitro*. It is possible that PKC isozymes other than the α, β and γ pool used in this study can phosphorylate these two sites more strongly. In

addition, selectivity of phosphorylation sites can be influenced in the cell by the intracellular localization of the kinases and the association of kinases and substrates with other proteins. Immunofluorescence studies have shown that PKC is associated with vimentin in interphase cells (Murti et al., 1992). Therefore, it seems that PKC may be a possible kinase responsible for vimentin hyperphosphorylation in the presence of fostriecin.

It is also important to note that although fostriecin, okadaic acid and calyculin A all inhibit PP1 and PP2A *in vitro* and cause hyperphosphorylation of vimentin and reorganization of intermediate filaments *in vivo*, okadaic acid and calyculin A have been recognized as potent tumour promoters (Fujiki et al., 1991; Haystead et al., 1989; Weinstein, 1991) whereas fostriecin is being tested in clinical trials as an antitumour agent (Moore et al., 1995; Schilsky et al., 1994). This suggests that fostriecin could also be a tumour promoter. It is thought that inhibition of protein phosphatases 1 and 2A causes the inactivation of the tumour suppressor proteins Rb and p53 by causing their sustained hyperphosphorylation (Fujiki, 1992).

Other tumour promoters such as phorbol esters are thought to act by activating PKC (Nishizuka, 1984; Wolfe et al., 1985) and 4β-phorbol 12-myristate 13-acetate has been shown to strongly stimulate vimentin phosphorylation in cultured human fibroblasts (Bertrand et al., 1994). This raises the possibility that despite having distinct primary targets, phorbol esters and protein phosphatase inhibitors might exert their tumour-promoting activity through activation of a common biochemical pathway. If this is the case, then vimentin hyperphosphorylation may be an early, readily detectable indicator of the activation of this pathway.

5.2 The importance of PP1 and PP2A in the regulation of cyclin levels.

In Chapter 4, we show that fostriecin induces premature degradation of cyclin A and cyclin B in human cancer cell lines. Although treatment of cells with fostriecin induces a certain percentage of cells to enter mitosis prematurely (Roberge et al., 1994), the degradation of cyclin induced by fostriecin is uncoupled from the process of mitosis. In the normal cell cycle, cyclins are rapidly degraded at the end of mitosis. At the fostriecin concentrations that were used in the degradation experiments, 10% of an asynchronous HeLa cell population entered mitosis prematurely (not shown) and >95% degradation of cyclins. Fostriecin causes degradation of cyclins even in interphase cells when cyclins are normally accumulating and very stable. These results suggest that the cyclin degradation pathway is present in interphase cells, but in a repressed form. This is supported by studies in amphibian interphase egg extracts which show that cyclin degradation can be triggered by adding cdc2 (Felix et al., 1990; Lorca et al., 1992), but that it is normally activated only at mitosis.

Various studies have implicated protein phosphatase 1 or 2A activity in the cyclin degradation pathway. In fission yeast, disfunctional mutants of either PP1 or PP2A affect entry and exit from mitosis (Yanagida et al., 1992). Without PP1, yeast cells remain blocked in mitosis with high H1 kinase activity, while in PP2A mutants, cdc2 is inactivated (Yanagida et al., 1992). This suggests that inactivation of PP2A but not PP1 in yeast affects the cyclin degradation pathway. Another group has shown that an okadaic acid-sensitive protein phosphatase negatively controls this degradation pathway in amphibian eggs (Lorca et al., 1991). Two possible interpretations are that PP1/PP2A plays a direct role in negatively regulating the cyclin degradation pathway during interphase or that PP1/PP2A plays an indirect role, for

example by negatively regulating a kinase that triggers cyclin degradation. Our preliminary studies show that specific kinases are activated in fostriecin-treated cells and this may play a role in cyclin degradation. The kinases and phosphatases involved in the cyclin degradation pathway still need to be identified. We showed that the activity of a staurosporine-sensitive kinase is required for fostriecin-induced cyclin degradation. This provides strong evidence that protein phosphorylation controls cyclin degradation in response to fostriecin. It will be important to identity the kinase to increase our understanding of this relatively unknown process.

The sudden proteolysis of proteins at specific times of the cell cycle is important for the control of the cell cycle. Other proteins involved in the cell cycle, such as p27, p53, jun and some oncogenes, are also degraded by the ubiquitin-proteasome pathway (Barinaga, 1995; Ciechanover and Schwartz, 1994). Here, I showed using proteasome inhibitors that fostriecin induces cyclin degradation through the ubiquitin-proteasome (Fig. 17). If fostriecin has the general effect of activating this degradation system, it would be interesting to determine whether the other cell cycle regulating proteins are degraded. This would exert profound effects on the cell cycle and may suggest other possible mechanisms by which fostriecin kills cells.

Since cyclins are essential for normal cell growth and division, induction of untimely cyclin degradation might be a significant contributor to fostriecin's cytotoxicity. We hypothesized that cells would not die if the proteasome was inhibited and untimely degradation of cyclins could not take place. Clearly this was an oversimplified view. The concentration at which fostriecin degrades nearly all cyclins also caused 100% cytotoxicity and proteasome inhibitors were not able to prevent cell death (Table 4). However, 100 μM fostriecin for 1 h causes 40-80% cell death without inducing cyclin

degradation and the proteosome inhibitor and cysteine protease inhibitors partially inhibited cell death (Table 4). It seems from these results that cyclin degradation is not essential for fostriecin's cytotoxicity, but that the degradation of other proteins by cysteine proteases is somehow required.

Apoptosis and necrosis are the two major forms of cell death. Necrosis is a pathologic form of cell death resulting from acute cellular injury, which is typified by rapid cell swelling and lysis. Apoptosis can be described as an internally encoded suicide program, which is activated by a variety of extrinsic and intrinsic signals. Apoptosis allows for the elimination of cells that have developed improperly or received genetic damage. Cells undergoing apoptosis are characterized by controlled autodigestion of the cell, involving the activation of endogenous proteases. This results in cytoskeletal disruption, cell shrinkage, membrane blebbing, condensation of nucleus and DNA fragmentation. Cancer chemotherapeutic agents are believed to cause aberrations in cellular physiology that induce apoptosis. In previous studies, fostriecin has been shown to induce morphological changes characteristic of apoptosis in HL-60 cells but not in MOLT-4 cells, which died by necrosis (Gorczyca et al., 1993; Hotz et al., 1992). My preliminary experiments suggest that fostriecin does not exclusively cause death of HeLa cells by necrosis or by apoptosis. Rather, 12-48 h after fostriecin treatment up to 10% of cells were necrotic and 10% of cells were apoptotic (not shown). It is interesting to note that cysteine protease inhibitors inhibit apoptosis in some cell lines (Thompson, 1995). The inhibition of proteases by the drugs used in this study may also prevent apoptosis and therefore, reduce cytotoxicity. However, more experiments should be carried out to determine the exact mechanism(s) by which fostriecin causes cell death. Different cell lines should be used since

the tendency to undergo apoptosis in response to particular treatments can differ among cell lines.

Cells normally appear to be able to detect dysregulation of proteins that control cell division, such as cyclins and cyclin-dependent kinases and to respond by apoptosis (Thompson, 1995). It is possible that premature induction of cyclin degradation or another imbalance in the cell cycle machinery is such a signal to trigger cell death. Since the inhibition of protein phosphatase activity by fostriecin would affect many pathways, it would not be surprising if such imbalances occurred. Cancer cells do not have the same regulatory and repair mechanisms as normal cells; fostriecin may selectively kill cancer cells over normal cells due to these differences. The key question that needs to be answered is: what are the primary pathways being affected by fostriecin under conditions for cancer treatment? This is not an easy task, but finding new targets for cancer therapy is a worthwhile goal. Even if fostriecin is found to be to an inviable cancer therapeutic drug, the study of fostriecin may lead to the design of better drugs and better methods of treatment.

5.3 Conclusion

The eukaryotic cell cycle is complex and tightly regulated. Disruptions or imbalances in the cell cycle can cause abnormal growth, leading to cancer. Modern cancer treatment relies on the ability of drugs to target selectively differences between cancer and normal cells. Most chemotherapeutic drugs target processes involving DNA, such as DNA replication or repair. The antitumour drug fostriecin targets protein phosphatase 1 and 2A, a novel property for a cancer therapy drug. This may be valuable in the treatment of some types of cancer.

I have found evidence that fostriecin is an *in vivo* inhibitor of protein phosphatase 1 and 2A. Treating BHK-21 cells with fostriecin induced the hyperphosphorylation of the intermediate filament vimentin. This effect is very similar to that of the known protein phosphatase 1 and 2A inhibitors okadaic acid and calyculin A. Fostriecin induced the hyperphosphorylation of vimentin at three tryptic peptides, two of which were sensitive to staurosporine. Furthermore, PKC phosphorylated these sites *in vitro*, suggesting that PKC likely plays a role in the hyperphosphorylation of vimentin. It should be noted that okadaic acid and calyculin A are tumour promotors while fostriecin is used as an antitumour drug. It is possible that fostriecin may also be a tumour promoter and this should be further studied.

The second part of my project involved trying to study the effects of inhibition of protein phosphatase activity on the cell cycle. In the human cancer cell lines studied, fostriecin induced premature degradation of cyclin A and cyclin B without a requirement for mitosis. The activity of staurosporine-sensitive kinases and the proteasome were found to be necessary for fostriecin-induced cyclin degradation. The identification of this unknown kinase(s) will be an important step in elucidating the pathways involved. The results suggest that protein phosphatase activity is required for the proper timing of cyclin degradation and that this degradation pathway is present even during interphase, but kept inactive by phosphatase activity. Upon treatment with fostriecin and inhibition of phosphatases, the degradation pathway is activated and cyclins are degraded. It would be interesting to determine whether other proteins which normally undergo degradation by the ubiquitin-proteasome pathway are prematurely degraded during fostriecin treatment.

We attempted to determine whether cyclin degradation is important for the cytotoxic effects of fostriecin, since cyclins are essential for cell cycle progression. Fostriecin's cytotoxicity was partially reduced not only by proteasome inhibitors, but also by other neutral cysteine protease inhibitors. These results suggest that premature cyclin degradation is not the primary pathway that triggers cell death, but that protein degradation is necessary, at least in part, for fostriecin cytotoxicity. Further research should concentrate on determining which primary pathway is involved in cell killing since this would lead to a better understanding of this drug and possibly to the design of better drugs or cancer therapy strategies.

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