The antitumor drug fostriecin induces vimentin hyperphosphorylation and intermediate filament reorganization

Duncan T.Ho and Michel Roberge

Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

Fostriecin is an antitumor drug in phase I clinical trials. We have recently shown that it is a potent inhibitor of protein phosphatases 1 and 2A in vitro, a property not previously described for an antitumor 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 protein phosphatase 1 and 2A inhibitors okadaic acid and calyculin A, which are also tumor promoters. Fostriecin induced vimentin hyperphosphorylation mostly at two sites, which were sensitive to staurosporine and could be phosphorylated by protein kinase C in vitro. Fostriecin-induced vimentin hyperphosphorylation also occurred in cells that lack p34cdc2 kinase activity. These results suggest that protein kinase C plays a direct or indirect role in vimentin hyperphosphorylation during exposure to fostriecin. The results also provide strong evidence that fostriecin inhibits protein phosphatases 1 and 2A in vivo and raise the possibility that it may have tumor-promoting activity.

Introduction

Fostriecin is an antitumor drug particularly active against leukemia, lung, breast and colon cancer cells in vitro and experimental tumors in mice (1), and is presently in phase I clinical trials (2,3). Fostriecin was thought to exert its cytotoxic effects through inhibition of topoisomerase II because it inhibits the purified enzyme with a 50% inhibitory concentration (IC50) of 40 μM (4). However, it was recently found that fostriecin does not inhibit topoisomerase II in crude cellular extracts (5) and that it does not prevent VM-26 from stabilizing covalent complexes between topoisomerase II and DNA in CCFR-CEM cells (6), suggesting that topoisomerase II is not a significant in vivo target. We have shown that fostriecin is a potent inhibitor in vitro of protein phosphatase 2A (PP2A) and, to a lesser extent, protein phosphatase 1 (PP1), but not protein tyrosine phosphatases (7). Fostriecin inhibits PP2A with an IC50 of 40 nM (7), 1000-fold lower than topoisomerase II, making PP2A a more plausible in vivo target for fostriecin. Since this is the first demonstration of an antitumor drug with specificity towards protein phosphatases, it is important to determine whether fostriecin inhibits protein phosphatases in living cells and to characterize its effects on protein phosphorylation.

Here we show that in living cells fostriecin induces the reorganization of vimentin intermediate filaments and the hyperphosphorylation of vimentin. Fostriecin stimulates phosphorylation at two sites, which are sensitive to the protein kinase C (PKC) inhibitor staurosporine and can be phosphorylated by PKC in vitro. These effects are very similar to those elicited by the structurally unrelated PP1 and PP2A inhibitors okadaic acid (8,9) and calyculin A (8,10,11), strongly suggesting that fostriecin targets protein phosphatases in vivo.

Materials and methods

Cell culture and drug treatments

BHK-21 cells were grown as monolayers in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum and antibiotics at 37°C in humidified 10% CO2. FT210 cells were grown in suspension in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics at 32°C in humidified 5% CO2 and were synchronized as described in (12). Fostriecin (NSC 339638, 94528) was obtained from the NCI as vials containing 25 mg of fostriecin, 39 mg ascorbic acid (as an antioxidant) and NaOH to neutralize to pH 7. Fostriecin was added to cells from a fresh stock in PBS. Okadaic acid was obtained from GIBCO as a 0.5 mM stock solution in 10% DMSO. Staurosporine (Sigma) was from a 5 μg/ml stock in DMSO.

Phosphorylation of proteins

[^32]P, (50 μCi/ml) was added to BHK-21 cells at the same time as the various drugs. The cells were harvested by trypan blue exclusion, pelleted and then lysed in 1/3 volume of 4XSDS sample buffer (200 mM Tris-HCl pH 6.8, 400 mM diethetritol, 8% SDS, 0.4% bromophenol blue, 40% glycerol). SDS-PAGE was performed according to (13). Equal amounts of protein extract were applied to each lane and the ^32P-labeled bands were visualized by autoradiography.

Isolation of intermediate filaments

Vimentin and desmin were isolated essentially as in (14). Briefly, cells were harvested, washed once with PBS and then lysed in isolation buffer (PBS, pH 7.4, containing 0.6 M KCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 50 U/ml micrococcal nuclease) at room temperature. This procedure solubilized the cellular constituents but not the intermediate filament caps, such as vimentin. Subsequent procedures were performed at 4°C to limit proteolysis. The intermediate filaments were collected by centrifugation (3000 g, 10 min), resuspended in isolation buffer (PBS, pH 7.4, containing 0.6 M KCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 50 U/ml micrococcal nuclease) at room temperature. After washing the membrane three times for 20 min each, it was incubated for 1 h with horseradish peroxidase-conjugated anti-mouse antibody (GIBCO) diluted 1:4000. Finally, the membrane was washed three times with PBS containing 1% Tween-20 and 0.1 mM phenylmethylsulfonyl fluoride. The pellet was solubilized in SDS sample buffer containing 8 M urea and then separated by SDS-PAGE using 8% polyacrylamide gels.

Western blotting and immunoprecipitation

Equal amounts of cellular protein were loaded per lane on 8% polyacrylamide gels. After electrophoresis, the proteins were transferred to a nitrocellulose membrane by electroblotting. The membrane was blocked with 10% milk Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20 supplemented with 3% non-fat dried milk for 1 h at room temperature. After washing the membrane three times for 20 min each, it was incubated for 1 h with horse-radish peroxidase-conjugated anti-mouse antibody (GIBCO) diluted 1:4000. Finally, the membrane was washed three times and vimentin was detected by enhanced chemiluminescence (Amersham).

Vimentin was immunoprecipitated from cell extracts essentially as in (15). Briefly, cells were harvested by trypsinization and lysed in 500 μl of lysis buffer (50 mM Tris-HCl pH 7.4, 5 mM ethylene glycol-bis-(Z-aminoethyl ether)N,N'-tetraacetic acid, 5 mM EDTA, 10 mM sodium pyrophosphate, 0.2% SDS). The samples were boiled for 10 min and then treated with DNase and proteinase K. The samples were then precipitated with acetone and redissolved in 2XSDS sample buffer (200 mM Tris-HCl pH 6.8, 400 mM diethetritol, 8% SDS, 0.4% bromophenol blue, 40% glycerol). SDS-PAGE was performed according to (13). Equal amounts of protein extract were applied to each lane and the ^32P-labeled bands were visualized by autoradiography.

Abbreviations: IC50, 50% inhibitory concentration; PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; PKC, protein kinase C; PKA, protein kinase A; MAP, mitogen activated protein.
I (0.5 μg/ml). Subsequent steps were performed at 4°C to minimize proteolysis. Cellular debris was removed by centrifugation at 10,000 g for 5 min. Triton X-100 (2%), 2 μg/ml leupeptin and 2 μg/ml pepstatin were then added to the supernatant. The samples were preclariﬁed with 20 μl of a 50% slurry of protein A-Sepharose beads for 1 h and then incubated overnight with 2.0 μg of vimentin antibody. Then 20 μl of a 50% slurry of protein A-Sepharose were added for 2 h with agitation. The beads were collected by centrifugation and washed three times with lysis buffer. The samples were analyzed by SDS–PAGE as described above.

**Phosphorylation of vimentin by PKC and protein kinase A (PKA)**

Vimentin and desmin were isolated from BHK-21 cells as described above. They were further puriﬁed by solubilization in disassembly buffer (0.1 M Tris–HCl pH 7.4, 25 mM β-mercaptoethanol, 8 M urea) and reassembly by dialysis against 5 mM Tris–HCl pH 7.4, 1 mM dithiothreitol, 1 mM ethyleneglycol-bis-β-aminoethylether-N,N′-tetraacetic acid, 0.17 M KCl. Vimentin and desmin were the only signiﬁcant proteins present in these puriﬁed samples, as assessed from Coomassie blue stained gels. Between 5 and 10 μg vimentin were incubated with 0.5 μg PKA catalytic subunit (Sigma) in 20 mM Tris–HCl pH 7.4, 1 mM dithiothreitol, 0.1 M NaCl, 0.05 mM ATP, 12 mM MgCl₂, 2 μCi/μl [γ-32P]ATP, at 30°C for 1 h. Between 5 and 10 μg vimentin were phosphorylated with 5 ng PKC (Promega) in 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid pH 7.4, 1 mM CaCl₂, 1 mM dithiothreitol, 10 mM MgCl₂, 1 mM ATP, 0.2 mg/ml phosphatidyl serine, 2 μCi/μl [γ-32P]ATP, at 30°C for 1 h. The reactions were stopped by addition of 1/3 volume of 4X SDS sample buffer.

**Tryptic peptide mapping and phosphoamino acid analysis**

Cells were prelabeled by incubation with 50 μCi/ml [32P]P, in phosphate-free medium supplemented with dialyzed fetal bovine serum for 4–8 h. After drug treatment for an additional 2 h, the cells were collected and vimentin was isolated as described above. Alternatively, vimentin phosphorylated by PKA or PKC in vitro was obtained as above. Vimentin and desmin were separated from one another using a preparative 8% polyacrylamide gel. The gel was stained with Coomassie blue and the upper vimentin band was excised. Vimentin was eluted from the polyacrylamide piece, digested with trypsin and analyzed by electrophoresis in pH 1.9 buffer (formic acid/acetic acid/water, 1:3:36) followed by chromatography in n-butanol/pyridine/acetic acid/water (15:10:3:12), as described in (16), using a Hunter Thin Layer Peptide Mapping System.

**Vimentin immunofluorescence**

BHK-21 cells were grown on poly-L-lysine-coated coverslips overnight. After drug treatment, the coverslips were centrifuged at 200 g for 2 min, rinsed with 10 mM sodium phosphate, 150 mM NaCl, fixed for 10 min in 3.7% paraformaldehyde in the same buffer and then washed twice with KB (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.1% BSA). The coverslips were then incubated with vimentin antibody dilute 1:7 in KB for 30 min at room temperature in a humididiﬁed chamber and then rinsed twice with KB. They were then incubated with CY3-conjugated goat anti-mouse IgG (Jackson Immunofluorescence Laboratory, Inc.) diluted 1:750 in KB for 30 min, washed and counterstained with the DNA dye bisbenzimide. Cells were photographed on Kodak Tmax 400 ﬁlm using a Zeiss Axiophot microscope.

All experiments were carried out at least three times except for those in Figures 3 and 8, which were performed twice.

**Results**

**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 [32P]P, for 2 h, cells were lysed in SDS sample buffer, and the proteins were separated by SDS–PAGE and analyzed by autoradiography. Fostriecin did not cause a general increase in protein phosphorylation over untreated cells (Figure 1). However, a single protein band at 55 kDa became hyperphosphorylated (Figure 1, lane 2). Incubation of cells with 0.5 μM okadaic acid, a known protein phosphatase inhibitor (17), resulted in a very similar pattern, with the hyperphosphorylation of a single 55 kDa protein (Figure 1, 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 okadaic acid solvent (Figure 1, lanes 1 and 3).

Phosphorylation of the 55 kDa protein was quantitated as a function of time after addition of fostriecin and [32P]P. It was detectable after 0.5 h and increased over the 2 h period (Figure 2A). Increased phosphorylation was detectable at 50 μM fostriecin and was maximal at ~200 μM (Figure 2B).

**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 protein (9–11). To determine whether the 55 kDa protein hyperphosphorylated by fostriecin was vimentin, extracts were made from cells treated with [32P]orthophosphate and ascorbic acid or fostriecin and subjected to immunoprecipitation with a monoclonal antibody to vimentin (Figure 3). As shown in Figure 3(A), the antibody immunoprecipitated the hyperphosphorylated 55 kDa protein from fostriecin-treated cells (Figure 3A, lane 2), but not from ascorbic acid-treated cells (Figure 3A, lane 1). The 32P-labeled 55 kDa band was...
Fostriecin and vimentin hyperphosphorylation

**Fig. 3.** The intermediate filament vimentin is hyperphosphorylated by fostriecin. Cells were incubated with 50 μCi/ml [32P]Pj and ascorbic acid (lane 1) or 200 μM fostriecin (lane 2) for 2 h at 37 °C. The intermediate filament vimentin was immunoprecipitated (A) or purified (B,C) from 32P-labeled 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 vimentin.

not detected when the vimentin antibody was omitted from immunoprecipitation reactions (not shown).

Intermediate filaments were purified from BHK-21 cells on the basis of their insolubility using the procedure of (14). Intermediate filaments from cells treated with [32P]Pj and ascorbic acid or fostriecin were analyzed by SDS-PAGE and autoradiography. Purified preparations contained two closely migrating protein bands—vimentin and desmin—at ~55 kDa (Figure 3B,D). Autoradiography showed that the hyperphosphorylated 55 kDa band was highly enriched in intermediate filament preparations from cells treated with fostriecin compared with preparations from cells treated with ascorbic acid (Figure 3C, lanes 1 and 2). Antibodies to vimentin reacted strongly with the 55 kDa protein in both preparations (Figure 3D). Electrophoretic separation of desmin and vimentin was achieved using longer gels; this showed that the phosphorylation of desmin was not stimulated by fostriecin treatment (not shown). Together these results demonstrate that the 55 kDa protein whose phosphorylation is stimulated by fostriecin is vimentin.

**Fostriecin causes morphological changes in vimentin intermediate filaments**

Microscopic examination of cells treated with 100 μM fostriecin showed that cells began to round up after 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 morphology of the intermediate filament network was 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 (Figure 4A). Mitotic control cells treated with ascorbic acid showed a diffuse staining indicative of vimentin depolymerization (Figure 4C). 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 (Figure 4B). Those in mitosis were similar to mitotic cells from control cultures.

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

As a first step in determining through which kinases fostriecin induces vimentin hyperphosphorylation, we identified the hyperphosphorylated amino acid residues. Vimentin isolated from cells treated with [32P]Pj and ascorbic acid or fostriecin was subjected to acid hydrolysis and phosphoamino analysis. Figure 5 shows that vimentin was phosphorylated exclusively at serine residues in controls (Figure 5A) and in fostriecin-
Fig. 5. Vimentin phosphorylation induced by ascorbic acid or fostriecin occurs only on serine residues. Cells were treated with 50 μCi/ml [32P]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 positions of the cold phosphoaminoc acid standards phosphoSer (s), phosphoThr (t) and phosphoTyr (y) are indicated. The arrow indicates the position of vimentin. Standards phosphoSer (s), phosphoThr (t) and phosphoTyr (y) are indicated. The arrow indicates the position of the phosphorylated serine residues visualized by autoradiography.

Fig. 6. Vimentin hyperphosphorylation is inhibited by staurosporine. Cells were treated with 50 μCi/ml [32P]orthophosphate and ascorbic acid (lanes 1 and 3) or 200 μM fostriecin (lanes 2 and 4) in the absence (lanes 1 and 2) or presence of 200 nM staurosporine (lanes 3 and 4). Cellular extracts were separated by SDS–PAGE and 32P was visualized by autoradiography. The arrow indicates the position of vimentin.

Fig. 7. Fostriecin-induced vimentin hyperphosphorylation does not require p34cdc2 kinase activity. FT210 cells were either maintained at the permissive temperature of 32°C (lanes 1 and 2) or arrested in G2 at the non-permissive temperature of 39°C (lane 3 and 4). The cells were then treated with 50 μCi/ml [32P]orthophosphate and ascorbic acid (lanes 1 and 3) or 100 μM fostriecin (lanes 2 and 4) for 2 h. Cells were collected, vimentin intermediate filaments were isolated and analyzed by SDS–PAGE and phosphorylated proteins were visualized by autoradiography. The arrow indicates the position of vimentin.

Treated cells (Figure 5B). No phosphorylated tyrosine or threonine residues were detected.

Next, we examined the sensitivity of vimentin hyperphosphorylation to Ser/Thr kinase inhibitors. The calmodulin antagonist W7 (10 μM), the calmodulin kinase II inhibitor KN-93 (10 μM) and the S6 kinase inhibitor rapamycin (50 nM) showed <10% inhibition (not shown).

Staurosporine is a potent inhibitor of p34cdc2 kinase (IC50 = 7 nM), mitogen-activated protein (MAP) kinases (IC50 = 7 nM) and PKC (IC50 = 9 nM). A 200 nM dose of staurosporine added at the same time as 200 μM fostriecin and 50 μCi/ml [32P]orthophosphate inhibited vimentin hyperphosphorylation by 70% (Figure 6, lane 4). About 30% of vimentin hyperphosphorylation was by staurosporine-insensitive kinases which were not studied further.

To investigate the role of p34cdc2 kinase in fostriecin-induced vimentin phosphorylation, we used a genetic approach and the inhibitor olomucine. The mouse FT210 cell line has a temperature-sensitive p34cdc2 allele. The cells grow normally at 32°C but at 39°C they lose their p34cdc2 kinase and are unable to enter mitosis (18). Fostriecin-stimulated vimentin phosphorylation was diminished in FT210 cells at 39°C (Figure 7, lane 4). The p34cdc2 kinase inhibitor olomucine (50 μM) showed <10% inhibition (not shown). These results indicate that the staurosporine-sensitive kinase responsible for 70% of the fostriecin-induced vimentin hyperphosphorylation is not p34cdc2 kinase. No specific inhibitors of MAP kinases are available. 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 ~50% inhibition (not shown).

Taken together, these results show that a staurosporine-sensitive Ser kinase is responsible for 70% of fostriecin-induced vimentin hyperphosphorylation. This kinase is unlikely to be p34cdc2 kinase but could be PKC.

Vimentin hyperphosphorylation at two staurosporine-sensitive sites phosphorylated by PKC in vitro

To examine whether PKC might be the kinase responsible for vimentin hyperphosphorylation, we carried out two-dimensional tryptic mapping of vimentin from cells treated with fostriecin or with fostriecin and staurosporine and compared the maps with those from vimentin phosphorylated by PKC in vitro. Figure 8(A) shows three major tryptic phosphopeptides (labeled 1–3) and five minor ones in vimentin isolated from cells treated with fostriecin alone. Figure 8(B) shows that the number of labeled phosphopeptides was unchanged by staurosporine treatment but that the intensity of labeling of phosphopeptides 1 and 3 was significantly reduced, by 60% and 65% respectively. This indicates that staurosporine-sensitive kinase(s) hyperphosphorylate vimentin at two sites during exposure to fostriecin.

Tryptic maps of vimentin phosphorylated by PKC in vitro showed eight phosphorylation sites (Figure 8C). Two of these corresponded to the major phosphopeptides 1 and 3 found in vivo and one corresponded to a minor phosphopeptide found in vivo (labeled 4). The other five sites were not present in vivo. Thus, PKC can phosphorylate the two sites that are...
Vimentin is an abundant protein in cells of mesenchymal origin and in many transformed cell lines (22), and is in a polymerized state visible as a filamentous network which spans the cytoplasm and provides tensile strength to the cells. At mitosis, vimentin becomes hyperphosphorylated, causing the filaments to depolymerize and form cytoplasmic aggregates (15,23,24).

The protein phosphatase inhibitors okadaic acid and calyculin A also cause vimentin hyperphosphorylation (8), disassembly and clustering of vimentin filaments around the nucleus (9) or in patches in the cytoplasm (10), and rounding up of the cells (9). Geisler et al. (21) 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 fostriecin concentrations at which we observed stimulation of vimentin phosphorylation within 2 h (50-200 μM, Figure 2B) are similar to the IC50 of 110-220 μM for several mammalian cancer cell lines during 1-2 h exposure (25, unpublished data), and about one order of magnitude higher than the peak plasma concentrations of >2 μM reported in clinical trials (2), but significantly higher than the IC50 of 0.5-10 μM for continuous exposure. Therefore, our observation that fostriecin stimulates vimentin hyperphosphorylation at 50 μM and above is compatible with the notion that fostriecin might exert its cytotoxic effects by inhibiting a protein phosphatase.

We recently showed that fostriecin can cause cells to enter mitosis prematurely, even when cells are normally arrested from doing so because their DNA is incompletely replicated or damaged (7). Is the vimentin hyperphosphorylation caused by fostriecin similar to that which occurs normally at mitosis? p34cdc2 kinase is the mitotic kinase that phosphorylates vimentin during mitosis and mediates structural rearrangements (15,23) and specifically phosphorylates Ser-55 in the N-terminal non-α-helical domain (24). We show that fostriecin can induce vimentin hyperphosphorylation even in cells that lack p34cdc2 kinase and in the presence of a p34cdc2 kinase inhibitor. The kinase(s) responsible for this hyperphosphorylation are probably interphase vimentin kinases rather than p34cdc2 kinase.

Vimentin phosphorylation by kinases active in interphase has been the subject of many studies (19-21,26-29). PKC and PKA phosphorylate vimentin efficiently in vitro at sites distinct from the mitotic phosphorylation site and are good candidates for interphase vimentin kinases (9). We are aware of no reports of vimentin phosphorylation by MAP kinases. We show that most of the fostriecin-induced vimentin hyperphosphorylation is due to a staurosporine-sensitive kinase that hyperphosphorylates two sites which can be phosphorylated by PKC in vitro. However, these were minor in vitro PKC phosphorylation sites (Figure 8). PKC comprises a family of at least 10 recognized isotypes. Thus, it is possible that PKC isotypes other than the α, β and γ pool used in this study can phosphorylate these two sites more strongly. In addition, in the cell itself, the phosphorylation site selectivity of kinases can be modulated by several factors such as the interaction of kinase or substrate with proteins. Immunofluorescence studies have shown that at least one PKC isotype (β) is associated with vimentin intermediate filaments in interphase cells (30). Alternatively, it is possible that the requirement for PKC in fostriecin-induced vimentin phosphorylation is indirect, by controlling the activity of another kinase. In summary, it seems likely that one or more PKC isotypes is required for vimentin hyperphosphorylation in the presence of fostriecin. Eriksson et al. (8) also found that calyculin A stimulates phosphorylation on the interphase sites and not on the mitotic sites of vimentin. Cyclic GMP-dependent

![Fig. 8. Tryptic phosphopeptide analysis of vimentin phosphorylated in vivo and in vitro.](https://academic.oup.com/carcin/article-abstract/17/5/967/2475716)
protein kinase can also phosphorylate vimentin in vitro (28,29). It could participate in the fraction of fosfotcin-induced vimentin phosphorylation that is not inhibited by the PKC-specific inhibitors calphostin C, UCN-01 and GF109203X.

Finally, it is noteworthy that although fosfotcin, okadaic acid and calcylin A all inhibit PP1 and PP2A in vitro and cause vimentin hyperphosphorylation and intermediate filament reorganization in vivo, okadaic acid and calcylin A have been recognized as potent tumor promoters (31–33), whereas fosfotcin is being tested in clinical trials as an antitumor agent (2,3). Fosfotcin could also be a tumor promoter. Other tumor promoters such as phorbol esters are thought to function by activating PKC (34,35) and 4β-phorbol-12-myristate-13-acetate has been shown to stimulate strongly vimentin phosphorylation in cultured human fibroblasts (36). This raises the possibility that despite having distinct primary targets, phorbol esters and protein phosphatase inhibitors might exert their tumor-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 a tumor-promoting pathway.

Acknowledgements

We thank Hilary Anderson for help with microscopy and for critical reading of the manuscript and Alison Buchan for use of her photomicroscope. This work was supported by a grant from the British Columbia Health Research Foundation to M.R. D.H. and M.R. hold a studentship and a scholarship respectively from the Medical Research Council of Canada.

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