Taxol-induced p34^{cdc2} Kinase Activation and Apoptosis Inhibited by 12-O-Tetradecanoylphorbol-13-acetate in Human Breast MCF-7 Carcinoma Cells

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Abstract

The p34^{cdc2} kinase is a highly regulated serine-threonine kinase that, when complexed with cyclins A and B, controls cell entry into mitosis. Recently, premature activation of p34^{cdc2} was shown to be required for apoptosis induced by a wide variety of agents. Here, we show that Taxol induced p34^{cdc2} kinase activity with a peak at 6 h in human breast carcinoma MCF-7 cells. We subsequently observed that the activation of CPP32/Yama protease as well as the cleavage of its substrate poly(ADP-ribose) polymerase occurred 9 h after Taxol treatment. Omouline, a potent p34^{cdc2} inhibitor, effectively prevented Taxol-induced p34^{cdc2} kinase activation and subsequent apoptosis. Furthermore, the treatment of cells with cyclin B1-specific antisense oligonucleotide also blocked Taxol-induced apoptosis, suggesting that cyclin B1-associated p34^{cdc2} kinase plays an important role in the induction of apoptosis by Taxol. 12-O-Tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activator, was found to exert strong protection against Taxol-induced cell death in MCF-7 cells. TPA inhibited Taxol-mediated activation of p34^{cdc2} kinase by preventing the dephosphorylation of the Tyr-15 residue on p34^{cdc2} without altering the levels of Cdc2 and cyclin B1. In contrast, the ability of Taxol to enhance tubulin polymerization was not inhibited by TPA. These findings suggest that modulation of protein kinase C signaling can protect against Taxol-induced cell death by inhibiting p34^{cdc2} kinase activation.

Introduction

Paclitaxel (Taxol) is an effective drug that shows encouraging activity in human ovarian and metastatic breast cancers (1) and malignant melanoma (2). The drug is an antimitotic agent whose action results in the formation of stable bundles of microtubules within cells (3). These effects of the drug are correlated with causation of mitotic and G_2-M arrest as well as cellular toxicity (4, 5). Although the interaction of Taxol with the cytoskeleton is well characterized, the molecular mechanisms by which such an interaction leads to cell cycle arrest and cell death are not well understood.

The p34^{cdc2} kinase is universally required as the master control enzyme during mitosis in eukaryotes (6). Its activity controls the G_2-M-phase transition by promoting breakdown of the nuclear membrane, chromatin condensation, and microtubule spindle formation. This kinase is activated by Thr-161 phosphorylation and its association with other proteins, primarily cyclin B. During G_2, Cdc25 phosphatase binds to and activates cyclin B/Cdc2 complex by dephosphorylating Cdc2 at phosphotyrosine 15 and phosphothreonine 14 residues, thus allowing catalysis of ATP within an ATP binding pocket (7, 8). Exit from mitosis requires the inactivation of p34^{cdc2} by degradation of the cyclin B-regulatory subunit (9) and dephosphorylation of p34^{cdc2} at Thr-161 (10). Recently, premature activation of p34^{cdc2} was reported to be required for apoptosis induced by a lymphocyte granule protease (11). An unscheduled and transient p34^{cdc2} activation was found in the apoptosis of HL-60 cells treated with anticancer drugs such as etoposide, camptothecin, and nitrogen mustard (12). Furthermore, treatment of HeLa cells with Taxol caused p34^{cdc2} activation that did not occur at the mitotic block but rather coincided with DNA fragmentation (13). These data strongly indicate that inappropriate activation of p34^{cdc2} may play an important role in Taxol-mediated apoptosis.

TPA,^3 an activator of PKC, was capable of modulating apoptosis induced by a wide variety of agents. TPA attenuated the expression of p53 as well as the induction of apoptosis in wild-type p53-transfected K562 cells treated with doxorubicin (14). In contrast, TPA treatment induced apoptotic cell death in androgen-sensitive human prostate LNCaP cells and human leukemic HL-60 cells (15, 16). These suggest that the critical point determining the fate of cellular response to TPA seems to depend on the type of cell and its state of differentiation or growth. However, the mechanisms

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Received 7/23/97; revised 9/30/97; accepted 10/31/97.

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^1 Supported by National Science Council Grant NSC 86-2621-B002-005-2.

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^3 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PARP, poly(ADP-ribose) polymerase; PKC, protein Kinase C; ICE, interleukin 1β-converting enzyme; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-aminomethyl coumarin.
by which TPA modulates the cell death program remain unclear.

It has recently been shown that TPA rapidly induced premature G2-M transition by inhibiting the protein kinase activity of the key component of mitosis-promoting factor, p34cdc2 (17). This raises the possibility that TPA may affect Taxol-mediated apoptosis by modulating p34cdc2 kinase activity. To address this issue, we sought to examine the possible effect of TPA on Taxol-induced p34cdc2 kinase activity and apoptosis in human breast MCF-7 cells.

**Results**

**Activation of p34cdc2 Kinase during Taxol-induced Apoptosis.** To investigate relationships between p34cdc2 kinase and Taxol-induced apoptosis, immunoprecipitates were prepared using anti-cyclin B1 antibody, and histone H1 kinase...
activity was measured from human breast cancer MCF-7 cells treated with Taxol at various time points. Fig. 1A shows a representative profile of p34\textsuperscript{cdc2} kinase activity from immunoprecipitates of asynchronized MCF-7 cells treated for up to 12 h with 1 \mu M Taxol. As quantitated in Fig. 1B, the kinase activity increased steadily, peaking at 6 h after Taxol treatment, and then decreased slowly to control levels by 12 h. To clarify whether or not the increase in Cdc2 kinase was due to the blockage in G\textsubscript{2}-M phase, we analyzed the cell cycle profile during Taxol treatment. As shown in Fig. 1C, Taxol did not cause any change in cell cycle progression during the first 9 h; however, the G\textsubscript{2}-M phase became evident after 12 h of Taxol treatment. We further examined the induction kinetics of apoptosis induced by Taxol using an ELISA-based quantitative assay that measures cytoplasmic histone-bound DNA complexes generated during apoptotic DNA fragmentation. Fig. 1D shows that the apoptosis induced by Taxol was initially detectable at 9 h (2–3-fold increase compared to untreated cells) and thereafter increased steadily to 8-fold by 24 h, as compared to control. Consistent with the apoptosis data, Taxol initially induced PARP cleavage at 9 h after treatment, as detected by using an anti-PARP antibody that recognizes the carboxyl-terminal of PARP protein (Fig. 1E). Also, Taxol induced CPP32/Yama protease activity with similar kinetics, as determined by using a fluorogenic tetrapeptide, Ac-DEVD-AMC (Fig. 1F).

Olomoucine, a specific p34\textsuperscript{cdc2} kinase inhibitor that has been found to compete the ATP-binding site of Cdc2 (18) was used here to confirm the involvement of p34\textsuperscript{cdc2} kinase in Taxol-mediated apoptosis. Treatment with 10 \mu M olomoucine (subcytotoxic dose) effectively inhibited Taxol-induced p34\textsuperscript{cdc2} kinase activity in MCF-7 cells (Fig. 2A). Taxol-mediated activation of CPP32/Yama protease as well as the onset of apoptosis was also blocked by olomoucine treatment (Fig. 2B and C). Because the expression of cyclin B1 is essential for p34\textsuperscript{cdc2} kinase activity, we used cyclin B1-specific antisense oligonucleotide to interrupt Cdc2 kinase and examined its effect on Taxol-mediated apoptosis. MCF-7 cells were treated with 10 \mu M cyclin B1-specific antisense oligonucleotide phosphorothioates for 12 h before the addition of 1 \mu M Taxol for another 16 h. Under this condition, the Taxol induction of apoptosis could obviously be prevented by the cyclin B1-specific antisense oligonucleotide phosphorothioates, but not by its sense oligonucleotide phosphorothioates (Fig. 3). These results suggest that p34\textsuperscript{cdc2} kinase activation indeed acts as a key regulator for Taxol-mediated activation of apoptosis.

**Inhibition of Taxol-induced Apoptosis by TPA**. To determine whether TPA would affect apoptosis induced by
Taxol in MCF-7 cells, we treated cells with 100 nM TPA for 1 h before Taxol treatment, and the extent of apoptosis was determined by morphological examination and ELISA assay. By 24 h after the 1 μM Taxol treatment, most of the cells showed typical apoptotic morphology manifested by cell rounding, blebbing, contraction, and, finally, detachment of cells from the dish. But no such cell death morphology was observed in untreated cells or in cells treated with TPA plus Taxol or in cells treated with TPA alone (data not shown). Consistently, we found that 0.1 and 1 μM Taxol caused a 4- and 9-fold increase in apoptosis by 24 h, respectively, compared to untreated MCF-7 cells, which was effectively prevented by treatment with 100 nM TPA (Fig. 4A). Additionally, TPA also exhibited an inhibitory effect on Taxol-induced CPP32/Yama protease activation (Fig. 4B) and PARP cleavage (Fig. 4C) in MCF-7 cells.

**Taxol-induced p34<sup>cdc2</sup> Activation Prevented by TPA.**

Next, we examined whether Taxol-mediated p34<sup>cdc2</sup> kinase activation would be affected by TPA in MCF-7 cells. As depicted in Fig. 5A, we found that TPA abolished Taxol-mediated activation of p34<sup>cdc2</sup> kinase. Under the same experimental conditions, TPA alone had no effect on p34<sup>cdc2</sup> kinase activity. Furthermore, Western blot analysis showed that TPA caused a decrease in p34<sup>cdc2</sup> kinase activity that was not due to reduction of the protein levels of p34<sup>cdc2</sup> or cyclin B1 (Fig. 5B). The phosphorylation status of p34<sup>cdc2</sup> at the Tyr-15 residue was determined using an antibody that specifically recognizes the Tyr-15-phosphorylated p34<sup>cdc2</sup>. Fig. 6 clearly shows that Taxol treatment resulted in a dephosphorylation of Tyr-15 on p34<sup>cdc2</sup> at 6 h of exposure that corresponded to the peak of p34<sup>cdc2</sup> kinase activation. Furthermore, TPA treatment prevented the dephosphorylation of Tyr-15 on p34<sup>cdc2</sup> induced by Taxol. The levels of protein tyrosine kinase Wee1 and protein tyrosine phosphatase Cdc25A were not changed during TPA or Taxol treatment (Fig. 6, middle and bottom). Because the apoptosis-inducing activity of Taxol is related to its ability to stabilize microtubules, we wished to determine whether TPA could interfere with the action of Taxol on microtubules. Using an intact cell assay, we found that TPA did not inhibit the ability of Taxol to stabilize polymerized microtubules or affect microtubule polymerization in MCF-7 cells (Fig. 7).

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Fig. 4. A, TPA prevents Taxol-induced apoptosis in MCF-7 cells. B, TPA attenuates Taxol-induced CPP32/Yama protease activity. MCF-7 cells were treated with either solvent control, 0.1 μM Taxol, 0.1 μM Taxol plus 100 nM TPA, 1 μM Taxol, 1 μM Taxol plus 100 nM TPA, or 100 nM TPA for 12 h. The apoptosis assay and CPP32 activity assay were described in "Materials and Methods." C, TPA blocks Taxol-induced PARP cleavage. The proteolytic cleavage of PARP was detected by anti-PARP antibody (Santa Cruz Biotechnology, Inc.) using Western blot. The means (bars, SD) were calculated from at least five independent experiments.

Fig. 5. A, TPA inhibits Taxol-induced p34<sup>cdc2</sup> kinase activation. MCF-7 cells were treated with 0.2% DMSO (control), 1 μM Taxol, 1 μM Taxol plus 100 nM TPA, or 100 nM TPA for 6 h, and then cyclin B1 immunoprecipitates were prepared for kinase assay. B, Western blot analysis of the level of cyclin B1 and Cdc2 in MCF-7 cells treated with 1 μM Taxol, 1 μM Taxol plus 100 nM TPA, or 100 nM TPA for 6 h by using anti-cyclin B1 or anti-Cdc2 antibody (Santa Cruz Biotechnology, Inc.).
Discussion

Here we report that Taxol induced an unscheduled activation of p34\textsuperscript{cdc2} kinase, which played an important role in the onset of apoptosis in human breast cancer MCF-7 cells. These findings support earlier observations that a Taxol lesion initiated apoptosis via regulation of the p34\textsuperscript{cdc2} kinase in HeLa S3 and NPC-TW01 cells (13, 19). It has recently been found that several different types of DNA damage could induce the unscheduled activation of p34\textsuperscript{cdc2} kinase activity in human leukemic HL-60 cells, which is prior to or commensurate with the induction of apoptosis (12). Our results, together with others (20), strongly suggest that premature activation of p34\textsuperscript{cdc2} kinase occurred in response to exogenous stresses in cells that were committed to apoptosis. The biological role of p34\textsuperscript{cdc2} activation in the process of apoptosis remains to be explored, but it has been proposed that this kinase could be involved in some of the mechanistic (morphological) changes of apoptosis.

Yao et al. (21) showed that ligation of Fas/APO-1-triggered apoptosis involves ICE proteolytic activity and activation of p34\textsuperscript{cdc2} kinase. Specific tetrapeptide inhibitors of ICE or CPP32 prevented the anti-Fas antibody-mediated activation of p34\textsuperscript{cdc2} and inhibited apoptosis (21). Their data indicated that Fas-mediated apoptosis required ICE/CPP32-mediated activation of p34\textsuperscript{cdc2} kinase and suggested that activation of p34\textsuperscript{cdc2} is a downstream determinant of apoptosis induced by ICE-related proteases. However, other data provided by Martin et al. (22) suggested that p34\textsuperscript{cdc2} is not a part of the apoptosis machinery but rather an upstream regulator of apoptotic machinery under certain conditions. In this case, we also found that the peak of p34\textsuperscript{cdc2} kinase activity occurred 3 h earlier than that of chromatin fragmentation and CPP32 protease activation induced by Taxol. Direct evidence showed that the treatment of cells with cyclin B1-specific antisense oligonucleotide blocked Taxol-induced apoptosis. Besides, olomoucine, a specific p34\textsuperscript{cdc2} kinase inhibitor, also effectively inhibited kinase activity at 6 h after Taxol treatment and subsequently blocked CPP32 proteolytic activity, PARP cleavage, and DNA fragmentation (as detected by ELISA assay). These findings strongly indicate that p34\textsuperscript{cdc2} may act as an upstream regulator for Taxol-mediated cytosine protease activation and the cell death process. Our data also provide evidence that activation of CPP32/Yama cytosine protease is a critical step during apoptosis induced by Taxol, and we have tried to clarify the possibility that Cdc2 regulated CPP32 activity via direct interaction between both proteins. However, we did not find a physical association between Cdc2 and pro-CPP32 using a communoprecipitation assay (data not shown). This suggests that other mediators may be involved in the activation of CPP32/Yama protease by p34\textsuperscript{cdc2}.

The role of PKC modulators in the regulation of apoptosis differs with cell types, a result due perhaps to the differential regulation of PKC isotypes with their separate and unique functions in different cells (23). Beyond describing the ability of PKC to modulate apoptosis, little is known of the mechanisms within either the PKC pathway or signaling cascades distal to PKC that are responsible for producing these disparate cell-specific responses. Here, we demonstrate that phorbol ester treatment provided strong protection for MCF-7 cells against Taxol-induced CPP32/Yama protease activation and DNA fragmentation. We suggest that the protective effect of TPA is exerted by preventing Taxol-induced p34\textsuperscript{cdc2} kinase activation that is possibly due to blockage of tyrosine dephosphorylation at Tyr-15. Our data, presented here, support the hypothesis that the TPA effect was not due...
to the change of protein levels of Cdc25A phosphatase or Wee1 kinase. Because we did not directly determine enzyme activity, the detail mechanism by which TPA prevented Taxol-mediated tyrosine dephosphorylation of Cdc2 remains to be investigated. Recently, Raf-1 has been found to associate with Cdc25A and thus stimulate Cdc25A phosphatase activity (24, 25). This raises the possibility that TPA treatment may interfere with the association between Cdc25A and Raf-1 by modulating PKC signaling.

On the other hand, we have shown that Taxol-enhanced tubulin polymerization was not suppressed by TPA during the inhibition of Taxol-induced apoptosis. The anticancer mechanism of Taxol was preliminarily considered to enhance polymerization or suppress the dynamic instability of microtubules, resulting in cell cycle blockage in G2-M and, ultimately, in cell death (26, 27). However, the phase of the cell cycle in which cancer cells underwent apoptosis differed, depending on exposure to low or higher concentrations of Taxol, i.e. the higher concentrations of Taxol induced homocycle apoptosis in the G2-M cells, but low doses or a short pulse probably led to their postmitotic apoptosis in the G1 phase (28, 29). In addition, Taxol has the ability to elicit apoptotic DNA fragmentation in both the G2-G1- and G2-M-synchronized cancer cells (30, 31). Our data provide one more piece of evidence to indicate that sustained microtubule stabilization, and hence persistent mitotic blockage, is not a requisite for Taxol-induced apoptosis.

**Materials and Methods**

**Cell Culture and Chemicals.** The human breast carcinoma MCF-7 cell line was cultured in RPMI 1640 containing 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 50 μg/ml streptomycin. Taxol (paclitaxel) and TPA were purchased from Sigma Chemical Co. The cyclin B1-specific antisense (5'-CATCGGCTTGAGGAGGATT-3') and sense (5'-AATTCCCTTCCAGGCGCGATG-3') phosphorothioate (S-oligo) were synthesized and purified by high-performance liquid chromatography (Genset Co.).

**Quantitation of Apoptosis by ELISA.** To quantitatively assay apoptotic cell death, we used an ELISA-based cell death assay kit (Boehringer Mannheim) that measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation as described by Sumantran et al. (32). Briefly, an anthistone first antibody was coated on wells, which were then loaded with cytoplasmic lysates (5 × 10⁵ cells/well). Cytoplasmic extracts from control-, Taxol-, or Taxol plus TPA-treated cells were equalized on the basis of total cell number. The second antibody was an anti-DNA antibody conjugated to peroxidase. The ELISA was developed with peroxidase substrate, and the absorbance at 405 nm was measured using a Microplate autoreader (EL311; Bio-tek Instruments, Winooski, VT).

**Assay of p34<sup>cdc2</sup> Histone H1 Kinase Activity.** Cell lysates were prepared for anti-cyclin B1 immunoprecipitation reactions as described previously (33). Briefly, 100 μg of lysates were incubated with 1 μg of anti-cyclin B1 antibody (Santa Cruz Biotechnology, Inc.) at 4°C for 1 h. Immunoprecipitates were then precipitated with 50 μl of protein A-Sepharose (Sigma Chemical Co.) overnight at 4°C, washed three times with NP40 lysis buffer by centrifugation and resuspension, and then washed twice with kinase buffer [50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, and 1 mM 1,1,1-trichloro-2,2-bis-(p-chlorophenyl)ethane]. The kinase assay was carried out by combining the washed Sepharose pellets with 45 μl of kinase buffer plus 10 μCi of [γ-³²P]ATP. Histone H1 (type Vb; Sigma) was added to a final concentration of 5 μg/ml. The reaction was allowed to proceed for 20 min at 37°C and was terminated by adding 30 μl of Laemmli sample buffer and boiling for 10 min. Products were resolved by 10% SDS-PAGE.

**Isolation of Polymerized Microtubules and Tubulin Dimers from Intact Cells.** Separation of polymerized tubulin from tubulin dimers and analysis of the effect of Taxol on tubulin polymerization in intact cells were performed as described by Blagosklonny et al. (34). In brief, MCF-7 cells were treated with either DMSO solvent control, 1 μM Taxol, 1 μM Taxol plus 100 μM TPA, or 100 μM TPA, and then treated cells were lysed in low-salt buffer [20 mM Tris-HCl (pH 6.8), 1 mM MgCl₂, 2 mM EGTA, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, and 0.5% NP40] and lysis samples were kept at room temperature under low ambient light to diminish the spontaneous polymerization of tubulin. After centrifugation of the lysates at 15,000 × g for 10 min to separate microtubules from soluble tubulin dimers, the microtubule pellets were resuspended in Laemmli's reducing loading buffer and heated at 95°C for 10 min to dissolve the pellets, and proteins were resolved on 8% SDS-polyacrylamide gels. After electrotransfer onto nitrocellulose membranes, the relative amounts of tubulins were detected by anti-α-tubulin monoclonal antibody (Oncogene Science, Inc.) and peroxidase-conjugated sheep antimouse secondary antibody. Bands were visualized by enhanced chemiluminescence (Amersham). The level of Cdc2 was detected by anti-cdc2 antibody as an internal control.

**Western Blot Analysis.** Protein samples were prepared in Laemmli's buffer and resolved by SDS-PAGE. The proteins were then transferred onto nitrocellulose membranes, and Western blot analyses were performed using rabbit polyclonal or mouse monoclonal antibodies specific to human PARP (Upstate Biotechnology, Inc.) CPP32/Tama, Cdc2, Cdc25, Wee1 (Santa Cruz Biotechnology), or tyrosin-15-phosphorylated p34<sup>cdc2</sup> (New England Biolabs). The goat antirabbit or antimouse antibody conjugated to horseradish peroxidase and enhanced chemiluminescence were used to visualize protein bands.

**Assay of CPP32 Proteolytic Activity.** CPP32 protease activity was measured by the method modified of Enari et al. (35). In brief, after cells (1 × 10⁶) were treated as indicated, cytosolic extracts were prepared by repeated cycles of freezing and thawing in 300 μl of extraction buffer [12.5 mM Tris (pH 7.0), 1 mM DTT, 0.125 mM EDTA, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin]. Cell lysates were diluted with the buffer [50 mM Tris (pH 7.0) 1 mM DTT, 0.5 mM EDTA, and 20% glycerol] and incubated at 37°C with 100 μM Ac-DEVD-AMC, CPP32 substrate. The fluorescent aminomethylcoumarin product was measured by a spectrofluorometer (Hitachi F-3000) using an excitation wavelength at 360 nm and an emission wavelength at 460 nm.

**References**


