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Resveratrol-induced G_2 arrest through the inhibition of CDK7 and $p34^{CDC2}$ kinases in colon carcinoma HT29 cells

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Abstract

Resveratrol (3,5,4′-trihydroxystilbene), a phytoalexin found in grapes and other food products, has been shown to have cancer chemopreventive activity. However, the mechanism of the anti-carcinogenic activity is not well understood. Here, we offer a possible explanation of its anti-tumor effect. Based on flow cytometric analysis, resveratrol inhibited the proliferation of HT29 colon cancer cells and resulted in their accumulation in the G_2 phase of the cell cycle. Western blot analysis and kinase assays demonstrated that the perturbation of G_2 phase progression by resveratrol was accompanied by the inactivation of $p34^{CDC2}$ protein kinase, and an increase in the tyrosine phosphorylated (inactive) form of $p34^{CDC2}$. Kinase assays revealed that the reduction of $p34^{CDC2}$ activity by resveratrol was mediated through the inhibition of CDK7 kinase activity, while CDC25A phosphatase activity was not affected. In addition, resveratrol-treated cells were shown to have a low level of CDK7 kinase-Thr^{161}-phosphorylated $p34^{CDC2}$. These results demonstrated that resveratrol induced cell cycle arrest at the G_2 phase through the inhibition of CDK7 kinase activity, suggesting that its anti-tumor activity might occur through the disruption of cell division at the G_2/M phase.

Keywords: Resveratrol; Cell cycle; $p34^{CDC2}$; CDK7; CDC25A

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene, Fig. 1) is a bioflavonoid found in many plants, including grapes and mulberries. In the world of plants, resveratrol, regarded as an antibiotic, is thought to play an important role in the host defense mechanism against infection and injury [1].

Resveratrol possesses many biological activities that can conceivably offer protection against atherosclerosis. These include antioxidant activity, modulation of hepatic apolipoprotein and lipid synthesis, inhibition of platelet aggregation, as well as its induction of the production of anti-atherogenic eicosanoids by human platelets and neutrophils [2]. Resveratrol has also been found to possess cancer chemopreventive activity through the inhibition of ribonucleotide reductase and cellular events associated with cell proliferation, tumor initiation, promotion, and progression [3–5]. Moreover, resveratrol has been found to be a cancer chemotherapeutic agent as it can decrease tumor growth in a rat tumor model and trigger CD95 signaling-dependent apoptosis in HL60 cells [6,7]. In addition, the action of resveratrol can be compared to non-steroidal anti-inflammatory drugs such as aspirin and sulindac, as it acts through the inhibition of cyclooxygenase activity [8,9]. We have demonstrated that resveratrol inhibits inducible nitric oxide synthase protein expression through the down-regulation of nuclear factor κB activity [10].

The $p34^{CDC2}$ protein kinase is generally acknowledged to be the key mediator of G_2/M phase transition in all eukaryotic cells [11,12]. The active mitotic kinase (MPF, or mitosis-promoting factor) is a dimer comprised of a catalytic subunit, $p34^{CDC2}$, and a regulatory subunit, a B-type cyclin [13–15]. The cyclins are a class of proteins that are synthesized during the interphase of each cell cycle and rapidly degraded at the end of mitosis [16]. The activity of the $p34^{CDC2}$ kinase not only depends on its association with cyclin B, but also on its phosphorylation state. Phosphorylation of either Thr^{14} or Tyr^{15} inhibits $p34^{CDC2}$ kinase

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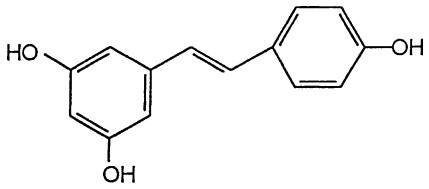

Resveratrol
(3,5,4'-trihydroxystilbene)

Fig. 1. Structures of resveratrol.

activity [17,18], while phosphorylation of Thr^{161} by CDK7 kinase is required for kinase activity [19–23]. In addition, the dephosphorylation of Thr^{14} and Tyr^{15} by CDC25A phosphatase is a final step for $p34^{CDC2}$ kinase activity [24–28].

Previous studies have shown that many flavonoids exhibit potent anti-tumor activity through cell cycle disruption. For example, genistein is able to cause a G_2/M arrest in several human and murine cell lines [28–31], and quercetin has been shown to block the G_1- to S-phase transition in a human gastric cancer cell [31]. Here, we report that resveratrol induces G_2 arrest and the inactivation of $p34^{CDC2}$ in colorectal carcinoma HT29 cells. Additionally, we show that resveratrol inactivates $p34^{CDC2}$ through the inhibition of CDK7 kinase activity and that this inhibition is independent of CDC25A phosphatase activity.

2. Materials and methods

2.1. Materials

Resveratrol, propidium iodide (PI), and RNase A were obtained from the Sigma Chemical Co. Isotope was obtained from Amersham. Stock solutions of resveratrol were prepared in DMSO and stored in the dark at −20°. The DMSO concentration in all drug-treated cells was 0.1%.

2.2. Cell culture

HT29, a colorectal carcinoma cell line, was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and kept at 37° in a humidified atmosphere of 5% CO_2 in air.

2.3. Growth assay

Assessment of proliferation was done as described [32]. HT29 cells were seeded into 96-well plates and grown for 18 hr. The cells were then treated with resveratrol for 24 or 48 hr, after which viability was assayed with a Cell Titer 96® Non-Radioactive Cell Proliferation Assay Kit (Promega). Briefly, 20 μL of a combined solution of a tetrazolium compound, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H* tetrazolium: inner salt], and an electron coupling reagent, phenazine methosulfate, was added to each well. The absorbance at 490 nm ($A_{490\ nm}$) was recorded using an ELISA plate reader, after a 2-hr incubation at 37° in a humidified 5% CO_2 atmosphere. For thymidine incorporation assay, cells were seeded into 24-well plates for 18 hr, and then treated with resveratrol for 24 hr, followed by the determination of [^{3}H]-thymidine (1 μCi/mL) incorporation into DNA during the last 4 hr of treatment. For the colony growth assay, cells were seeded in triplicate at a density of 1000 cells per well and grown for 10 days in the absence or presence of drug. The resulting colonies were fixed, stained with 0.25% methylene blue in 50% methanol/water, and counted. Colonies composed of at least 50 cells were scored. The colony-forming efficiency was determined by dividing the number of colonies by the number of cells plated. All experiments were done at least twice using triplicate samples.

2.4. Immunoprecipitation and in vitro kinase assay

Cell lysates were prepared for immunoprecipitation and the kinase assay was performed as described by Huang *et al.* [33] with some modification. The $p34^{CDC2}$ kinase, CDK7 kinase, and CDC25A phosphatase were immunoprecipitated over an 18-hr period at 4° with $p34^{CDC2}$, CDK7, and CDC25A-specific antibodies (Santa Cruz Biotechnology), respectively, and protein-A/G agarose. Then the immunoprecipitates were reacted with their substrate, histone H1 (Calbiochem), in kinase buffer consisting of 20 mM Tris–HCl (pH 7.4), 7.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μM ATP, and 10 μCi [γ-^{32}P]-ATP. The reaction was terminated by boiling the samples for 10 min in 5× SDS–PAGE loading buffer. Proteins were separated by 10% SDS–PAGE. The gels were subsequently dried and autoradiograped −70°.

2.5. Western blot analysis

Equal amounts of total cellular protein (50 μg) were resolved by SDS–PAGE, and transferred to Immobilon-P membranes (Millipore) as described previously [34]. The membranes then were incubated with one of the following antibodies: anti-$p34^{CDC2}$ monoclonal (Santa Cruz Biotechnology), anti-phospho(Tyr^{15})-specific $p34^{CDC2}$ monoclonal, anti-phospho(Thr^{161})-specific $p34^{CDC2}$ polyclonal (New England Biolabs), or anti-cyclin B1, anti-cyclin A, anti-Wee1, or anti-GAPDH polvclonals (Santa Cruz Biotechnology). The immunocomplexes were detected by enhanced chemiluminescence (Amersham), or incubation with colorigenic substrates nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as suggested by the manufacturer (Sigma).

2.6. Flow cytometric cell analysis

Cell cycle distribution was analyzed by flow cytometry as described previously [34]. Briefly, cells were trypsi-

nized, washed once with PBS, and fixed in 100% ethanol for 1 hr at −20°. Fixed cells were washed with PBS, incubated with 0.5 mL PBS containing 0.05% RNase and 0.5% Triton X-100 for 30 min at 37°, and stained with PI. The stained cells were analyzed using a FACScan laser flow cytometer (Becton Dickinson).

3. Results

3.1. Effects of resveratrol on the inhibition of cell proliferation

To assess the effect of resveratrol (Fig. 1) on the proliferation of colon cancer cells, we first determined the growth rates of HT29 colon cancer cells. Exponentially growing HT29 cells were cultured continuously in the absence or presence of 50 or 100 μM resveratrol, and cell growth was evaluated at 24 and 48 hr with the MTS assay. Resveratrol strongly inhibited cell growth (Fig. 2A). DNA synthesis in HT29 cells was determined by the incorporation of [^{3}H]-thymidine. In resveratrol-treated cells, the incorporation of [^{3}H]-thymidine was about 50% of that in control cells and DMSO-treated cells (Fig. 2B). We also examined the effect of resveratrol on the growth of HT29 cells at low seeding densities by the colony growth assay. HT29 cells were plated, treated with resveratrol or DMSO for 10 days, and were fixed, stained, and quantified using this assay. The colony growth assay confirmed the effectiveness of resveratrol in dramatically reducing the growth of HT29 cells (Fig. 2C).

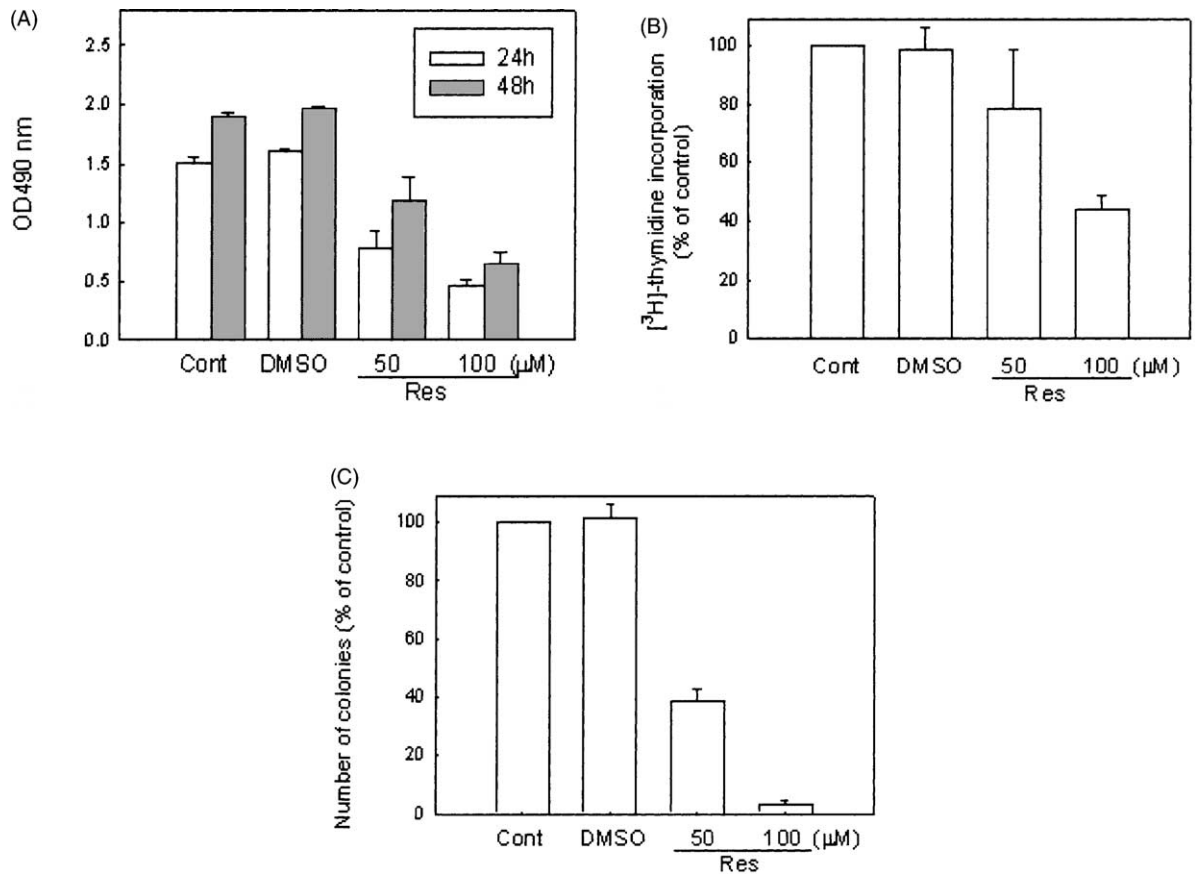

Fig. 2. Growth arrest in resveratrol-treated HT29 cells. (A) Cells were treated with resveratrol (Res) or DMSO (0.1%) for 24 and 48 hr, and cell growth was determined by the MTS assay as described in Section 2. The experimental data were obtained in two (N = 2) or three (N = 3) independent experiments. All experiments were performed in duplicate, and results are shown as means ± SEM. Control, 24 hr, 1.50 ± 0.06, N = 2; 48 hr, 1.89 ± 0.04, N = 3. DMSO, 24 hr, 1.61 ± 0.0, N = 3; 48 hr, 1.96 ± 0.03, N = 2. Resveratrol (50 μM), 24 hr, 0.78 ± 0.16, N = 3; 48 hr, 1.19 ± 0.20, N = 3. Resveratrol (100 μM), 24 hr, 0.46 ± 0.05, N = 2; 48 hr, 0.65 ± 0.10, N = 3. (B) Cells were treated with resveratrol or DMSO for 24 hr and were labeled with [^{3}H]-thymidine for 4 hr. The radioactivity of each sample was analyzed as described in Section 2. The [^{3}H]-thymidine values of the control were about 2052 cpm. The relative [^{3}H]-thymidine incorporation of other treatments was presented as follows: DMSO, 98.70 ± 7.50, N = 2. Resveratrol (50 μM), 78.25 ± 20.86, N = 2. Resveratrol (100 μM), 43.70 ± 5.23, N = 2. The results were obtained in two (N = 2) or three (N = 3) independent experiments performed in duplicate and are shown as means ± SEM. (C) Cells were seeded in 6-well plates and grown for 10 days in the presence of resveratrol or DMSO. The number of colonies was quantified as described in Section 2. The colony numbers of the control were about 152. The results were obtained in two (N = 2) or three (N = 3) independent experiments performed in duplicate and are shown as means ± SEM. The relative colony numbers of the other treatments were as follows: DMSO, 101.50 ± 4.95, N = 2; resveratrol (50 μM), 38.85 ± 4.03, N = 2; resveratrol (100 μM), 3.00 ± 1.41, N = 2.

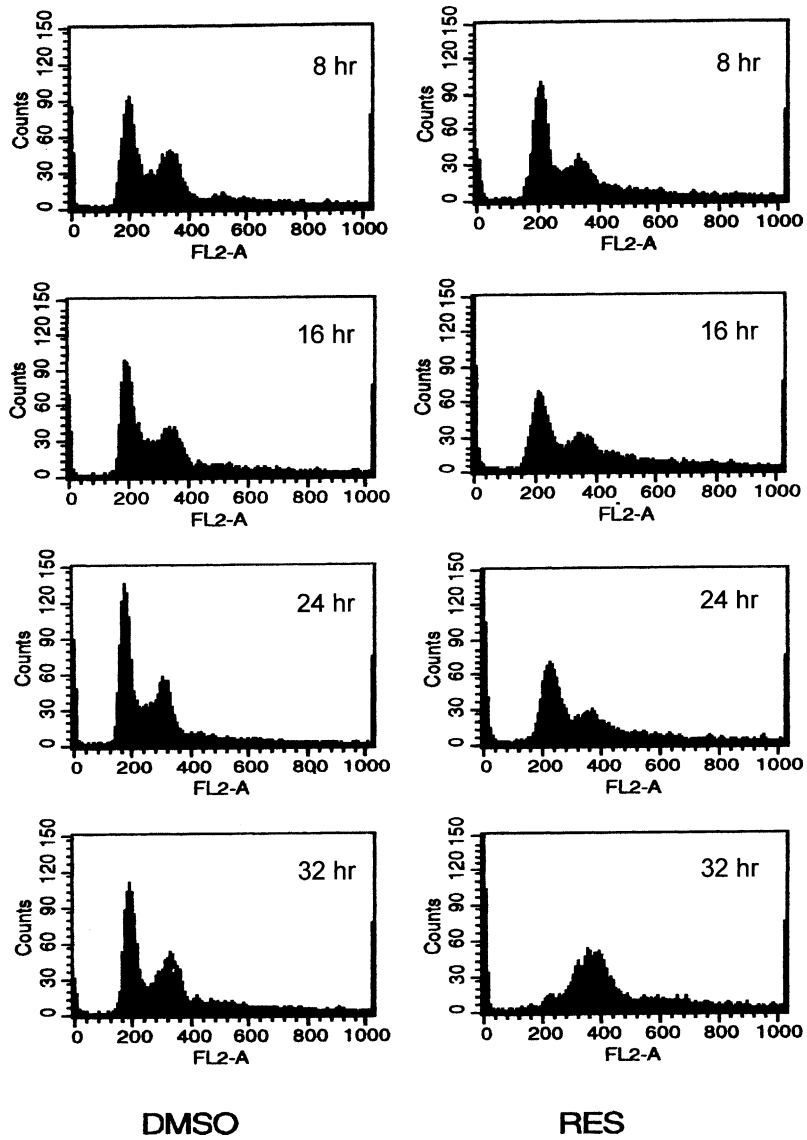

Fig. 3. Effects of resveratrol on the cell cycle progression. Cells were treated with DMSO (0.1%) or resveratrol (100 μM) for the indicated time, and cell cycle analysis was performed as described in Section 2. Data shown are representative of at least three independent experiments.

3.2. G_2 phase arrest in resveratrol-treated cells

To test whether resveratrol could affect the cell cycle of HT29 cells, asynchronized cells treated with DMSO or resveratrol for 8, 16, 24, and 32 hr were subjected to flow cytometric analysis after DNA staining. Histograms of flow cytometric data are shown in Fig. 3. Control cells (DMSO) progressed through the cell cycle. In contrast, resveratrol-treated HT29 cells were blocked in the G_2/M phase after a 32-hr treatment. The nuclei were enlarged and no mitotic figures were observed, suggesting that the cells were blocked in the G_2 rather than the M phase (data not shown).

3.3. Inhibition of $p34^{CDC2}$ protein kinase in resveratrol-treated cells

We next examined the changes of G_2/M regulatory proteins in resveratrol-treated HT29 cells. The cells were treated with or without resveratrol (l00 μM) for 12, 24, or 36 hr, and then protein levels were determined by western blot analysis. As shown in Fig. 4A, the levels of cyclin A and cyclin B1 gradually decreased in the DMSO-treated control cells. In contrast, in resveratrol-treated cells cyclin A started to accumulate at 12 hr and peaked at 24 hr, whereas cyclin B1 was not degraded and reached maximal levels at 36 hr. Both Wee1 and Myt1 are dual-specificity protein kinases that phosphorylate the Thr^{14} and Tyr^{15} residues of $p34^{CDC2}$. The protein levels of Wee1 (Fig. 4A), Myt1 (data not shown), and $p34^{CDC2}$ (Fig. 4B) were not changed in resveratrol-treated cells compared to the respective controls. Since $p34^{CDC2}$ kinase is the key regulator that promotes mitosis, we further examined the effect of resveratrol on $p34^{CDC2}$ kinase activity. Cyclin B1/$p34^{CDC2}$ complexes were isolated by immunoprecipitation from HT29 cell extracts treated with DMSO (0.1%) or resveratrol (100 μM) for 12, 24, and 36 hr, and the kinase activity was measured with histone H1

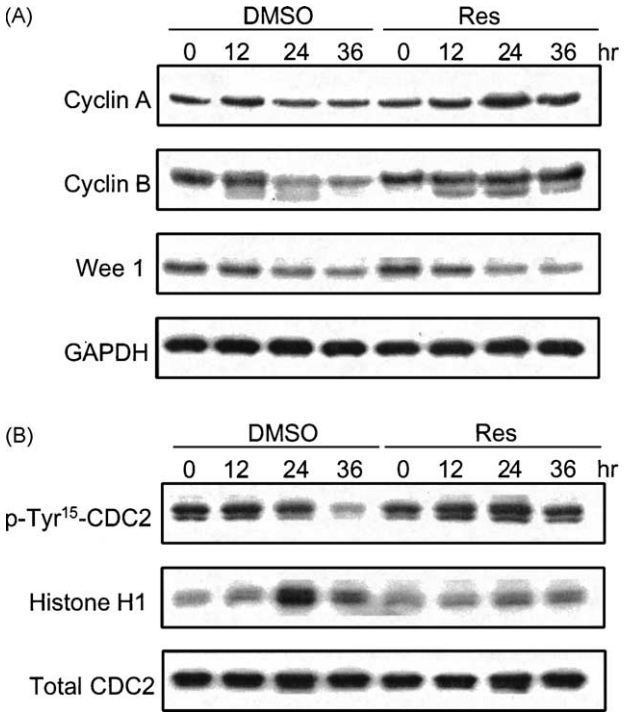

Fig. 4. Effects of resveratrol on the cyclin proteins and $p34^{CDC2}$ kinase activity. HT29 cells were treated with DMSO (0.1%) or 100 μM resveratrol (Res) for 0, 12, 24, and 36 hr. (A) Western blot analyses were done with anti-cyclin A, anti-cyclin B1, anti-Wee1 and anti-GAPDH primary antibodies as described in Section 2. (B) Western blots with anti-$p34^{CDC2}$ and anti-phospho(Tyr^{15})-specific $p34^{CDC2}$ antibodies (upper and lower panels); $p34^{CDC2}$ kinase activity assay with histone H1 as substrate (middle panel). Data shown are representative of at least three independent experiments.

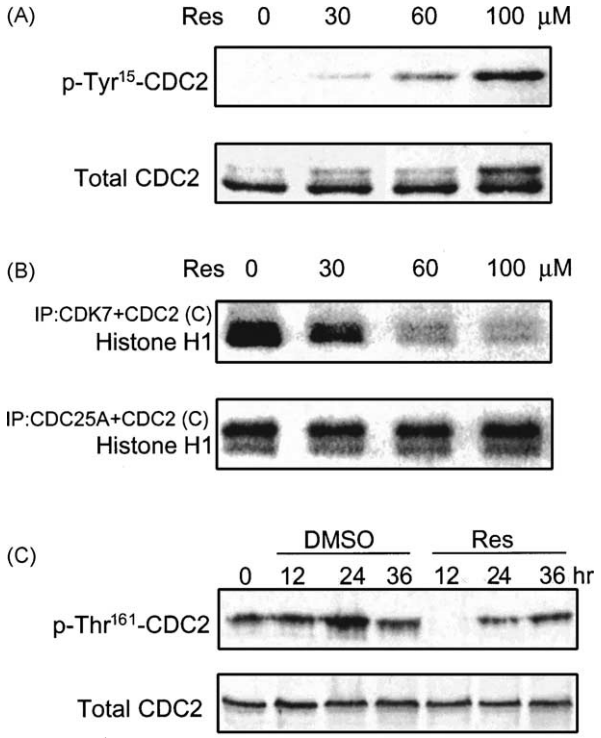

Fig. 5. Effects of resveratrol on CDK7 kinase and CDC25A phosphatase activities. (A) Western blot analysis of the phospho-Tyr^{15}-$p34^{CDC2}$ level in HT29 cells treated with 30, 60, or 100 μM resveratrol (Res) for 32 hr using an anti-phospho(Tyr^{15})-$p34^{CDC2}$ or anti-$p34^{CDC2}$ monoclonal antibody. (B) Cells were treated with 30, 60, or 100 μM resveratrol (Res) for 32 hr, and total protein was collected. CDC25A kinase and CDK7 kinase activities were assayed as described in Section 2. (C) Cells were treated with 0.1% DMSO or 100 μM resveratrol (Res) for 0, 12, 24, and 36 hr. Western blot analyses were done using an anti-phospho(Thr^{161})-$p34^{CDC2}$ antibody as described in Section 2. Data shown are representative of at least three independent experiments.

as substrate. As shown in Fig. 4B, maximum $p34^{CDC2}$ kinase activity was observed in control cells (DMSO) at 24 hr, which corresponded to the M phase of the cell cycle. Resveratrol prevented the activation of $p34^{CDC2}$ kinase as compared to the DMSO-treated cells. Since tyrosine dephosphorylation has been shown to be tightly linked to the activation of $p34^{CDC2}$, and since $p34^{CDC2}$ kinase was not activated by resveratrol treatment, we assessed the possibility that $p34^{CDC2}$ dephosphorylation at Tyr^{15} may also have been inhibited. Total cell lysates prepared from cells treated with resveratrol for 12, 24, and 36 hr were immunoblotted with anti-$p34^{CDC2}$-phospho(Tyr^{15}) antibody. A 36-hr exposure to resveratrol prevented the dephosphorylation of $p34^{CDC2}$ at Tyr^{15} (Fig. 4B) in a concentration-dependent manner (Fig. 5A). These results suggested that resveratrol might first trigger the inhibition of $p34^{CDC2}$ tyrosine dephosphorylation, which could then lead to the prevention of $p34^{CDC2}$ kinase activation.

3.4. Effects of resveratrol on CDK7 kinase and CDC25A phosphatase activities

To further investigate the underlying reasons for the prevention of $p34^{CDC2}$ kinase activity in HT29 cells treated with resveratrol, we examined the possible involvement of two known $p34^{CDC2}$ regulators, CDK7 kinase and CDC25A phosphatase. For these experiments, CDK7 kinase and CDC25A phosphatase were immunoprecipitated from the extracts of HT29 cells exposed to 30, 60, or 100 μM resveratrol or 0.1% DMSO. These CDK7 kinase and CDC25A phosphatase preparations were each mixed with $p34^{CDC2}$ immunoprecipitates from untreated HT29 cells in exponential growth. Following incubation, the mixtures were examined for the inhibition of $p34^{CDC2}$ kinase activity using an *in vitro* kinase assay. Results of the experiments revealed that the CDK7 kinase preparations from resveratrol-treated cells were able to reduce $p34^{CDC2}$ kinase activity (Fig. 5B, top panel) in a concentration-dependent manner, whereas the CDC25A phosphatase preparations from resveratrol-treated cells showed no effect on $p34^{CDC2}$ kinase activity (Fig. 5B, bottom panel). Western blot analysis for CDK7 and CDC25A indicated that the level of CDK7 and CDC25A proteins was not changed (data not shown). In addition, the level of Thr^{161}-phosphorylated $p34^{CDC2}$ increased at 24 hr and then decreased to the basal level at 36 hr in the DMSO-treated control cells. However, exposure to 100 μM resveratrol for 12 hr prevented the phosphorylation of $p34^{CDC2}$ at Thr^{161}.

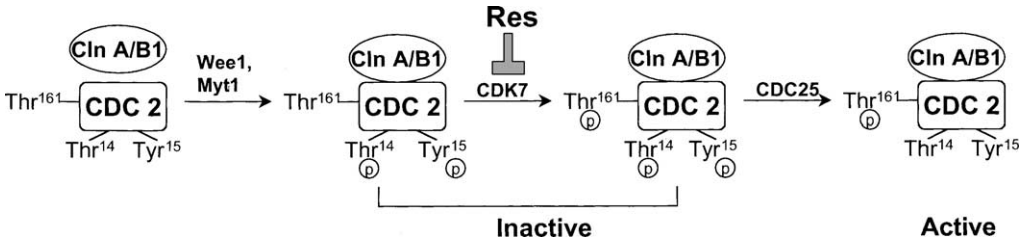

Fig. 6. Inhibition by resveratrol of $p34^{CDC2}$ regulation (modified from Ref. [33]). Activation of $p34^{CDC2}$ kinase includes several steps: $p34^{CDC2}$ is first phosphorylated on Thr^{14} and Tyr^{15} residues by Wee1 kinase and the related Myt1 and forms a complex with cyclin B1; the inactive complex then is phosphorylated on Thr^{161} by CAK (cyclin H/CDK7/MAT1 complex); this pre-active, tyrosine-phosphorylated cyclin B1/$p34^{CDC2}$ complex is bound and fully activated by CDC25A phosphatase through the dephosphorylation of $p34^{CDC2}$ on Thr^{14} and Tyr^{15} residues. At the end of mitosis, the active $p34^{CDC2}$ is down-regulated by dissociation of the cyclin B1/$p34^{CDC2}$ complex. The data in this report demonstrate that resveratrol could affect $p34^{CDC2}$ kinase by inhibiting the activity of CDK7 protein kinase.

Even at 36 hr Thr^{161}-phosphorylated $p34^{CDC2}$ was at basal levels (Fig. 5C). These findings indicated that the inhibiting effect of resveratrol on $p34^{CDC2}$ kinase activity was mediated through the inhibition of CDK7 kinase. Thus, we concluded that resveratrol treatment prevents the exit of HT29 cells from the G_2 phase by its disruption of CDK7 kinase activity and resulting inactivity of $p34^{CDC2}$ kinase.

4. Discussion

Colorectal carcinoma is a common cause of death by cancer. It has been estimated that about 70–90% of colon cancer death can be linked to diet [35,36]. Recently, there is considerable interest in using dietary prevention and chemoprevention to decrease mortality. Dietary factors contribute to one-third of the potentially preventable cancers, and the long known protective effect of a plant-based diet on tumorigenesis and other chronic diseases is also documented [37,38]. Resveratrol is a bioflavonoid found in many plants, including grapes and mulberries. Furthermore, red wine is believed to be the main source of resveratrol in the human diet. In this study, we have clearly shown that resveratrol was able to inhibit the proliferation of colorectal carcinoma HT29 cells. Lu and Serrero [39] also have indicated that resveratrol exhibits an inhibitory effect on human breast cancer cells. These results suggested that resveratrol might be an effective natural component for cancer chemoprevention.

As revealed by flow cytometry (Fig. 3), HT29 cells treated with resveratrol accumulated in the G_2/M phase of the cell cycle. Because there was no evidence of an increased percentage of mitotic cells in resveratrol-treated cultures, upon microscopic examination, the observed accumulation in G_2/M indicates cell arrest in G_2 rather than in mitosis. This finding is consistent with previous work, which showed that resveratrol could induce HL60 cells and bovine endothelial cells to arrest in the G_2 phase [40,41]. Furthermore, resveratrol prevented the dephosphorylation of Tyr^{15} of the $p34^{CDC2}$ kinase which resulted in kinase inactivation (Fig. 4). Since the hyperphosphorylation of the kinases responsible for phosphorylation of $p34^{CDC2}$ at Thr^{14} and Tyr^{15} is one of the known mechanisms for G_2 arrest [42], these results indicated that resveratrol could block the cell cycle in the G_2 phase and offered an explanation for why the HT29 cells could not proliferate. This is consistent with the hypothesis that the cyclin-dependent kinase required for the G_2 to M transition might be a target of the flavonoids.

To better understand the mechanism by which resveratrol decreased the activity of $p34^{CDC2}$ kinase, we explored the effects of resveratrol on the regulation of $p34^{CDC2}$ activity. Fig. 6 shows that activation of $p34^{CDC2}$ can be controlled at several steps, including at the phosphorylation/dephosphorylation of $p34^{CDC2}$ at Thr^{161}, Thr^{14}, and Tyr^{15} residues [42]. In this study, we have shown for the first time that resveratrol is able to inhibit $p34^{CDC2}$ kinase activity. As described in Fig. 6, the activity of $p34^{CDC2}$ was dependent upon CDK7 kinase and CDC25A phosphatase activities. Further experiments indicated that CDK7 activity was inhibited by resveratrol and failed to phosphorylate Thr^{161} of $p34^{CDC2}$ (Fig. 5) but the Thr^{14} and Tyr^{15} residues of $p34^{CDC2}$ remained phosphorylated. This inactive form of $p34^{CDC2}$ failed to advance the cells from the G_2 phase into mitosis and caused them to arrest in the G_2/M transition. Previous studies have indicated that resveratrol is a remarkable inhibitor of ribonucleotide reductase, cyclooxygenase 1, and inducible nitric oxide synthase [3,4,10]. Here, our results showed for the first time that resveratrol was an inhibitor of CDK7 kinase. This property of resveratrol could explain why resveratrol could inhibit the proliferation of different kinds of cells.

In conclusion, we have shown that resveratrol inhibits HT29 cell proliferation by disturbing CDK-7 kinase activity, which causes $p34^{CDC2}$ kinase to remains inactive and the cells to arrest in the G_2 phase of the cell cycle. These effects of resveratrol are compatible with its putative chemopreventive and/or anti-tumor activity.

Acknowledgments

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