Natural Product Triptolide Mediates Cancer Cell Death by Triggering CDK7-Dependent Degradation of RNA Polymerase II

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Abstract

Triptolide is a bioactive ingredient in traditional Chinese medicine that exhibits diverse biologic properties, including anticancer properties. Among its many putative targets, this compound has been reported to bind to XPB, the largest subunit of general transcription factor TFIIH, and to cause degradation of the largest subunit Rpb1 of RNA polymerase II (RNAPII). In this study, we clarify multiple important questions concerning the significance and basis for triptolide action at this core target. Triptolide decreased Rpb1 levels in cancer cells in a manner that was correlated tightly with its cytotoxic activity. Compound exposure blocked RNAPII at promoters and decreased chromatin-bound RNAPII, both upstream and within all genes that were examined, also leading to Ser-5 hyperphosphorylation and increased ubiquitination within the Rbp1 carboxy-terminal domain. Notably, cotreatment with inhibitors of the proteasome or the cyclin-dependent kinase CDK7 inhibitors abolished the ability of triptolide to ablate Rpb1. Together, our results show that triptolide triggers a CDK7-mediated degradation of RNAPII that may offer an explanation to many of its therapeutic properties, including its robust and promising anticancer properties. *Cancer Res; 72(20); 5363–73. ©2012 AACR.*

Introduction

Triptolide is a principal bioactive ingredient of Tripterygium wilfordii Hook F, which has been used to treat autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and psoriasis in China (1). In addition to its unique structure of a diterpenoid triepoxide, triptolide has aroused extensive research interests due to the following pharmacologic characteristics. Triptolide possesses multiple apparently unrelated activities, including anti-inflammatory, immunosuppressive, antifertility, anticystogenesis, and anticancer activities (1), which seem to suggest multiple molecular mechanisms involved in those activities. Moreover, according

to a comparative analysis using the NCI-60 internal database, the GI$_{50}$ profile of triptolide is significantly different from those of 118 reference anticancer compounds (2), suggesting that triptolide could have anticancer mechanisms and molecular targets potentially distinct from those of other anticancer agents. Consistent with its various activities, triptolide has been revealed to interact with several cellular proteins, including polycystin-2 (PC-2), disintegrin and metalloprotease 10 (ADAM10), dCTP pyrophosphatase 1 (DCTPP1), and XPB (1). These proteins with distinct functions have thus been identified as its potential primary targets. And possibly, more potential targets would be added to the list in future because using the known targets of triptolide cannot fully explain its activities. Triptolide inhibits RNA polymerase II (RNAPII)–dependent transcription, showing a distinct mode of action from those of other known transcriptional inhibitors, including 5,6-dichloro-1-β-D-ribofuranosylbenz-imidazole (DRB) and flavopiridol (CDK9 inhibitors), actinomycin D (a DNA binder), and α-amanitin (an RNAPII inhibitor; refs. 2–5). Triptolide is thus considered to be a new type of transcriptional inhibitors (4).

The effect of triptolide on transcription has been paid special attention in recent years because this effect seems to contribute in most of its biologic activities, in particular, anticancer, anti-inflammatory, and immunosuppressive activities (1). Triptolide affects mRNA transcription in a concentration- and time-dependent manner. Treatment with triptolide at low concentrations and/or short exposure largely inhibits the transcription of short-lived mRNA, and possibly as a compensatory response, increases the transcription of some other

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genes such as *p53*. In contrast, its high concentrations and persistent exposure could reduce 80% mRNA transcription (2). Two factors, XPB inhibition and RNAPII degradation induced by triptolide, have been proposed to be responsible for its transcriptional inhibition (1, 6). XPB, a subunit of the general transcription factor TFIIH, is involved in both RNAPII-mediated transcription initiation and DNA nucleotide excision repair (NER; ref. 7). Triptolide directly binds to XPB, inhibits its ATPase activity and consequently, represses RNAPII transcription initiation and NER (4, 6). The previous reports revealed that inhibition of both XPB activity and mRNA transcription required only low concentrations of and/or short exposure to triptolide but induction of RNAPII degradation needed relatively high concentrations of and/or long exposure to the drug (2, 4, 6). Those data seem to suggest that RNAPII degradation induced by triptolide could contribute to its transcriptional inhibition at a relatively later stage but to a relatively more severe degree than XPB inhibition. Therefore, clarifying the molecular mechanism(s) of RNAPII degradation induced by triptolide would be critical to fully understand its transcriptional inhibition.

Degradation of RNAPII often follows ubiquitination of its largest subunit, Rpb1 (8–10). Ubiquitination can occur at different sites, both on the carboxy-terminal domain (CTD; ref. 11) and other residues (12), which depends on the type of transcriptional stress or stalling. The CTD consists of multiple repeats of the consensus sequence $Y_1S_2P_3T_4S_5P_6S_7$, plays a fundamental role in regulating the RNAPII-mediated transcription, and the stability of Rpb1 (8, 13, 14). Phosphorylation at Ser-5 and Ser-2 of the CTD regulates transcription initiation and elongation, respectively, at specific steps (14, 15). Ser-5 phosphorylation is mainly catalyzed by cyclin-dependent kinase 7 (Cdk7), a subunit of the general transcription factor TFIIH, which occurs in early phases of transcription initiation and allows promoter clearance of RNAPII. In contrast, Ser-2 is mainly phosphorylated by the positive transcriptional elongation factor (PTEF-b; the CDK9/cyclin T1 complex), which promotes transcriptional elongation (14). On the basis of the inconsistent data from different experimental approaches in the previous reports (8, 10, 16–18), however, whether phosphorylation of the CTD is correlated with ubiquitination and RNAPII degradation remains controversial and has to be clarified.

In terms of triptolide, particularly, although Rpb1 degradation by this agent has been consistently reported, whether it elicits phosphorylation of the CTD also seems controversial (3, 4, 6). In addition, although the inability to recruit TFIIH has been shown to induce RNAPII stalling at promoters (19, 20), whether triptolide could induce an RNAPII stalling at active promoter regions remains to be established. Moreover, XPB inhibition by triptolide is known to suppress transcription initiation (4) and as known, only a fraction of the total RNAPII binds to template DNA and engages in transcription (10). However, whether triptolide-induced Rpb1 degradation affects the chromatin-bound RNAPII is yet to be investigated.

Our current study was designed to answer those open questions. Following validation of the triptolide-induced Rpb1 degradation, our results revealed that this agent elicited

hyperphosphorylation of Rpb1 at Ser-5 of the CTD via CDK7, which contributed to its RNAPII degradation. We further found that the chromatin-bound reduction of Rpb1 at promoters was subsequent to a block of RNAPII at promoter-proximal sites of transcribed genes. Our data also showed that the Rpb1 degradation was associated with the cell-killing potency of triptolide, indicating the biologic importance of the triptolide-driven, CDK7-mediated Rpb1 degradation.

Materials and Methods

Reagents

Triptolide, used in our experiments, was prepared from Tripterygium wilfordii Hook F as described previously (21). MG132, epoxomicin, cycloheximide (CHX), DRB, and H-7 were purchased from Sigma. Triptolide (10 mmol/L), MG132 (10 mmol/L), epoxomicin (10 mmol/L), CHX (50 mg/mL), and DRB (50 mmol/L) were dissolved in dimethyl sulfoxide (Sigma) as stock solutions, respectively; H-7 (50 mmol/L) was dissolved in ethanol. The stock solutions were kept in aliquot at −20°C (triptolide, epoxomicin, CHX, DRB, and H-7) or at −80°C (MG132) and thawed immediately before each experiment. RPB1 (CTD repeat), phospho-Ser-2-RPB1, phospho-Ser-5-RPB1, and ubiquitin antibodies were from Abcam, CDK7, and β-actin antibodies were from Cell Signaling Technology, RPB1 (H-224) antibody was from Santa Cruz Biotechnology, and GAPDH antibody was from Beyotime.

Cells and culture conditions

Human cancer cell lines MKN-45 (gastric), A431 (epidermoid carcinoma), HeLa (cervical), A549 (lung), DU145 (prostate), HepG2 and BEL-7402 (liver), SK-OV-3 (ovarian), A375 (malignant melanoma), and HCC1937 and MCF-7 (breast) were obtained from the cell bank of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, PR China). Human cancer cell lines PC-3 (prostate), 786-O (kidney), and MDA-MB-436 (breast) were obtained from the American Type Culture Collection. PC-3 cells were authenticated with the Promega Cell ID System by BMR-Genomics. Beijing Microread Gene Technology Co, Ltd. used short tandem repeat profiling to authenticate all other cell lines. All the cells were also periodically authenticated by morphologic inspection and tested Mycoplasma contamination. Cells were cultured in Dulbecco's Modified Eagle's Medium; A431, HeLa, HepG2, SK-OV-3, A375, and MCF-7), F12 (A549 and PC-3), L-15 (MDA-MB-436), or RPMI-1640 (DU145, BEL-7402, MKN-45, 786-O, and HCC1937) medium (Life Technologies) supplemented with 10% FBS (Life Technologies) at 37°C in a humidified atmosphere containing 5% CO_2.

Growth inhibition assays

The growth inhibition of triptolide was examined by sulforhodamine B (SRB) assays as described previously (21). Briefly, cells in 96-well plates were treated in triplicate with gradient concentrations of triptolide at 37°C for the indicated time, and then assessed with SRB (Sigma). The absorbance at 560 nm was detected with a plate reader (SpectraMax; Molecular Devices). The survival rate was calculated as (A_{560} treated/A_{560} control) × 100%.

Cancer Research

siRNA transfection

PC-3 cells were plated at 18,000 cells/cm^2 density. Transfection was conducted 24 hours after the plating with the RNAi-Max transfection reagent and 20 nmol/L of scramble siRNA or CDK7 siRNA (Invitrogen). CDK7 silencing testing and triptolide treatments were conducted 72 hours after transfection.

Western blotting analyses

Cells were lysed in 1 × SDS lysis buffer [50 mmol/L Tris-HCl (pH 6.8), 100 mmol/L DTT, 2% SDS, 0.1% bromphenol blue, and 10% glycerol] and then boiled for 10 minutes. Western blotting analyses were conducted as previously described using appropriate antibodies and the levels of cellular proteins were visualized with peroxidase-coupled secondary antibodies (Dingguo) using an ECL Plus Kit from Amersham Biosciences.

Reverse transcription-PCR analyses

Cells were treated with triptolide for the indicated time. Total RNA was isolated with the TRIzol reagent. Total RNA was reverse transcribed using Superscript III reverse transcriptase and cDNA was used for PCR with the following primers (synthesized by Sanggon Corporation): *β-actin*, 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′ (forward), 5′-CTA GAA GCA TTG CGG TCG ACG ATG GAG GG-3′ (backward); *RPB1* primer 1, 5′-CAA GTC TAT GGT CGT GTC CG-3′ (forward), 5′-CCT TGA TGA AGT GAG GCA GA-3′ (backward); *RPB1* primer 2, 5′-CAA GTC TAT GGT CGT GTC CG-3′ (forward), 5′-CAT CCT TGA TGA AGT GAG GC 3′ (backward); *RPB1* primer 3, 5′- TGC CAC AGA CAG ACA ACA AGA AG -3′ (forward), 5′-TGA CAT AGG AGC CAT CAA AGG AG-3′ (backward). Amplification was done for 35 cycles, each with denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. The products were analyzed using agarose gel electrophoresis and visualized by ethidium bromide staining.

Chromatin immunoprecipitation analyses

Chromatin preparation and chromatin immunoprecipitation (ChIP) procedures have been described previously (22, 23). In brief, cells were fixed with 1% (v/v) formaldehyde for 15 minutes at 20°C, and chromatin was sonicated to an average DNA fragment size of 300 to 400 bp. Chromatin was equilibrated in RIPA buffer, and samples were precleared with nonimmune IgG. Equal amounts of precleared chromatin were then incubated with specific antibodies against the first 224 amino acids of the N-terminal domain of the largest subunit of RNAPII or preimmune IgG (to measure background recovery). Immunoprecipitated DNA was purified after proteinase K treatment and phenol extraction. Specificity of PCR products was routinely controlled by melting curve analyses and agarose gel electrophoresis. DNA recovery was determined at promoter–proximal regions from 200 bp upstream to 100 bp downstream from transcription start site and at coding exons of *c-myc*, *hif1-α*, *polr2a*, *vegf* genes, and α-satellite. DNA recovery was measured by quantitative real-time PCR with LightCycler (Roche Diagnostics) and normalized to control cells. The used primers were: *c-myc* P2 promoter, AGAAGGGCAGGGCTTCT-CAGA and TCTGCCTCTCGCTGGAATTAC; *c-myc* exon2,

TAGCTTCACCAACAGGAACT and AGCTCGAATTTCTTCCA-GAT; *c-myc* exon3, AGCCACAGCATACATCCTGTC and CTC-AGCCAAGGTTGTGAG GTT; *hif1-α* promoter, AGCTCCTCA-GTGCACAGTGC and GACTAGAGAGA AGCGGGCG; *hif1-α* exon2, AGCCAGATCTCGGCGAAGTA and CCAGAAGT TT-CCTCACACGC; *vegf* promoter, TTTAAAAGTCGGCTGGTA-GCG and GA AGAGGGATAAAACCCGGATC; *vegf* exon1, GC-TTGGGGAGATTGCTCTAC TTTC and CAGGTCACTCACTT-TGCCCCCT; *vegf* exon3, GCTACTGCCAT CCAATCGAGAC and CATGGTGATGTTGGACTCCTCAG; *polr2a* promoter, GG-GGAGAGACAAACTGCCGTA and GGGGAAATAAGGAGCGA-AAGG; and α-satellite, AGACACAAGCATTCTCAGCAA and CTTTTTCATCATAGGCCTCAA.

Statistical analyses

Data were presented as mean ± SD, and statistical significance was assessed with the Student *t* test. Differences were considered significant at P < 0.05.

Results

Phosphorylation at Ser-5 of the CTD is an early molecular event before Rpb1 reduction induced by triptolide

To confirm whether triptolide can induce reduction of Rpb1 as reported (2, 4, 6) and to clarify its kinetics, we treated human ovarian (SK-OV-3) and prostate (PC-3) cancer cells with 1,000 nmol/L triptolide for the indicated time. The Western blotting results showed that the treatment led to persistent Rpb1 reduction, beginning at the 30-minute time-point in SK-OV-3 cells and at the 40-minute time-point in PC-3 cells (Fig. 1A). In response to the similar treatment, interestingly, the levels of phosphorylation at Ser-5 of the CTD first rose before 15 minutes, reached their maximum at 30 minutes, and then began to decrease in SK-OV-3 cells (Fig. 1B). The result was repeatable in PC-3 cells when exposed to a lower concentration of triptolide (100 nmol/L) for longer times (Fig. 1C). At the same condition, however, the levels of phosphorylation at Ser-2 remained unchanged in both SK-OV-3 and PC-3 cells and the levels of Rpb1 itself reduced persistently (Fig. 1B and C). The data suggest that the selective induction of phosphorylation at Ser-5 of the CTD could be an early molecular event before the Rpb1 decrease, and the latter is possibly responsible for the subsequent reduction of the phosphorylated Rpb1 at Ser-5 in the triptolide-treated cancer cells.

The tumor cell–killing activity of triptolide correlates with its RNAPII decrease

To determine whether the effect of triptolide on RNAPII is a critical event for cytotoxic activity, we detected by Western blotting and observed the differential degradation of the cellular Rpb1 protein in a panel of cancer cell lines exposed to gradient concentrations of triptolide for 6 hours (Fig. 1D and Supplementary Fig. S1A). Triptolide induced Rpb1 decrease in a concentration-dependent manner, and 100 nmol/L was the lowest concentration, leading to a significant RNAPII reduction in the majority of the examined cell lines under the studied conditions. Among those cell lines, human gastric MKN-45, and ovarian SK-OV-3 cancer cell lines represented the ones
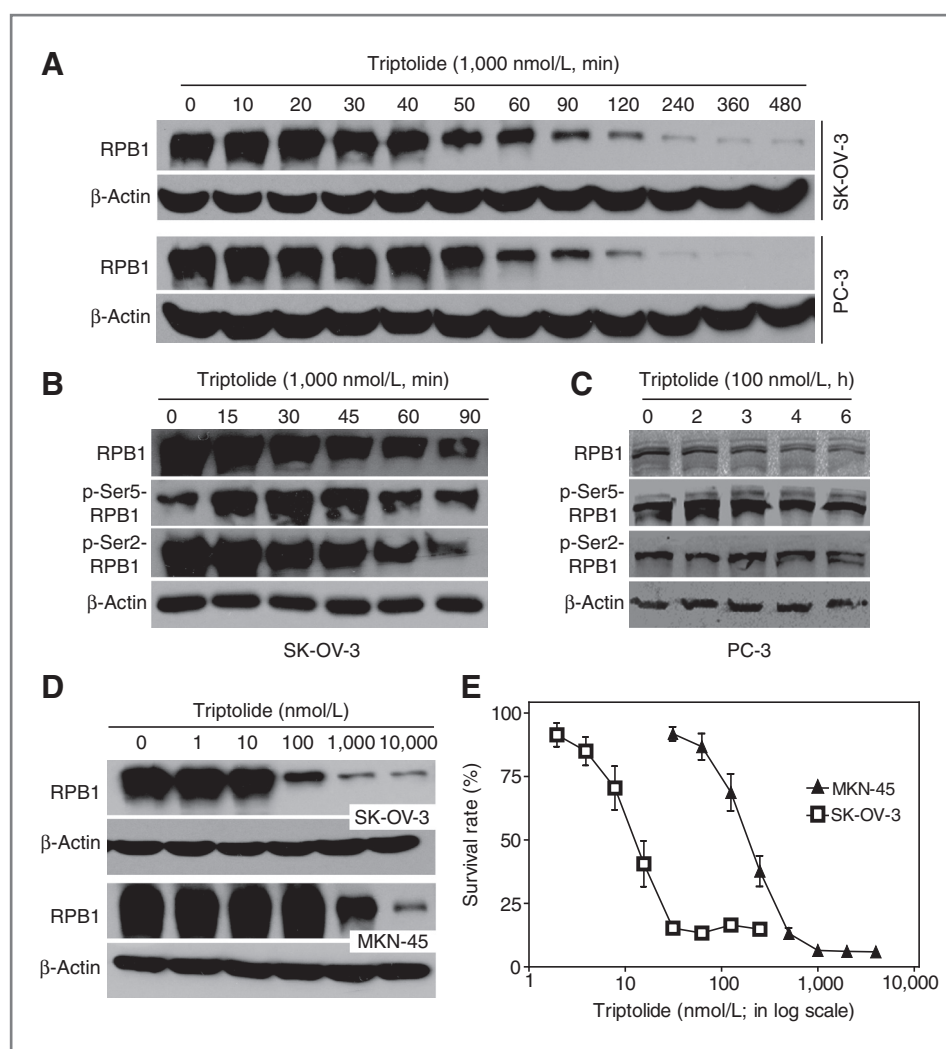
Figure 1. Triptolide induced Ser-5 hyperphosphorylation before RNAPII decrease, which associated with its antiproliferative activity. SK-OV-3 (A and B) and PC-3 (A and C) cells were exposed to triptolide as indicated. Nuclear proteins (20 μg) were then detected by Western blotting to assess the levels of Rpb1 and phosphorylated Rpb1 at Ser-2 or Ser-5 of the CTD using the indicated antibodies. D, SK-OV-3 and MKN-45 cells were treated with gradient concentrations of triptolide for 6 hours, and then harvested for Western blotting analyses to detect RPB1 levels. β-Actin was used as the loading control. E, the same cells were exposed to gradient concentrations of triptolide for 72 hours, and the cell survival was determined with SRB assays.

resistant and sensitive to the triptolide-induced Rpb1 reduction, respectively. Consistently, MKN-45 cells revealed much lower sensitivity to the tumor cell–killing effects of triptolide than SK-OV-3 (Fig. 1E and Supplementary Fig. S1B). The data indicate that the degree of the RNAPII decrease induced by triptolide could be correlated with its anticancer activity.

Triptolide-induced Rpb1 decrease involves a Cdk7-mediated proteasome-dependent protein degradation mechanism

As a general transcriptional inhibitor, triptolide can lead to reduction of cellular mRNA (2, 4). So we assessed whether the observed decrease of Rpb1 was attributable to the downregulation of its mRNA levels. RT-PCR analyses by using different amplicons along the Rpb1 mRNA sequence clearly revealed that Rpb1 mRNA levels did not change significantly in SK-OV-3 cells exposed to triptolide (Fig. 2A). Moreover, the triptolide-induced decrease of Rpb1 was independent of its new protein synthesis as shown by cotreatments with the protein synthesis inhibitor cycloheximide (CHX; Fig. 2B), excluding the possibility that the Rpb1 decrease resulted from its reduced protein

synthesis. Instead, the inhibition of proteasomal activity with either epoxomicin (Fig. 2C) or MG132 (Supplementary Fig. S2A) reversed the triptolide-induced decrease of Rpb1, showing that the latter involves a proteasome-dependent protein degradation mechanism. As expected, the treatment with 1 mmol/L triptolide for 20 minutes led to increased hyperphosphorylation at Ser-5 and ubiquitination of Rpb1 in SK-OV-3 cells (Fig. 2D). The von Hippel–Lindau tumor suppressor protein VHL and the tumor suppressor BRCA1 have been reported to ubiquitinate Rpb1 (11, 24). However, the triptolide-induced degradation of Rpb1 seemed to be independent of either of them because Rpb1 degraded apparently in VHL-deficient 786-O renal cancer cells and BRCA1-deficient MDA-MB-436 and HCC1937 breast cancer cells when exposed to triptolide (Supplementary Fig. S2B).

To investigate the possible correlation of this protein degradation mechanism with the induction of phosphorylation at Ser-5 by triptolide, we treated SK-OV-3 cells with the Cdk inhibitors DRB (50 μmol/L) and H7 (500 μmol/L). DRB has been shown to inhibit Cdk7 and Cdk9 with about 3-fold lower efficiency for the former, but H7 could inhibit both of them

Figure 2. The triptolide-induced RPB1 decrease is attributable to a Cdk7-mediated proteasome-executed protein degradation mechanism. A, SK-OV-3 cells were treated with triptolide for 6 hours and then harvested to detect the levels of RPB1 mRNA by reverse transcription-PCR analyses with 3 distinct Rpb1 primers. B, SK-OV-3 cells were treated with cycloheximide (CHX, 100 μg/mL), triptolide (1,000 nmol/L), or both and then the levels of Rpb1 protein in each sample were assessed by Western blotting. C, SK-OV-3 cells were exposed to triptolide with or without 10 μmol/L epoxomicin for 6 hours, and then the cells were harvested to test for the levels of Rpb1 protein by Western blotting. D, SK-OV-3 cells were exposed to triptolide (1 μmol/L) for 20 minutes, and then the cells were harvested for immunoprecipitation (IP) assays with an anti-RPB1 antibody. The levels of RPB1, p-Ser-5-RPB1, and ubiquitin in the RPB1-immunoprecipitated complexes or whole-cell lysates were tested by Western blotting. GAPDH was used as the loading control of the whole-cell lysates. E, SK-OV-3 cells were exposed to triptolide with or without DRB or H7 for 6 hours, and then the cells were harvested to test for the levels of Rpb1 protein by Western blotting.
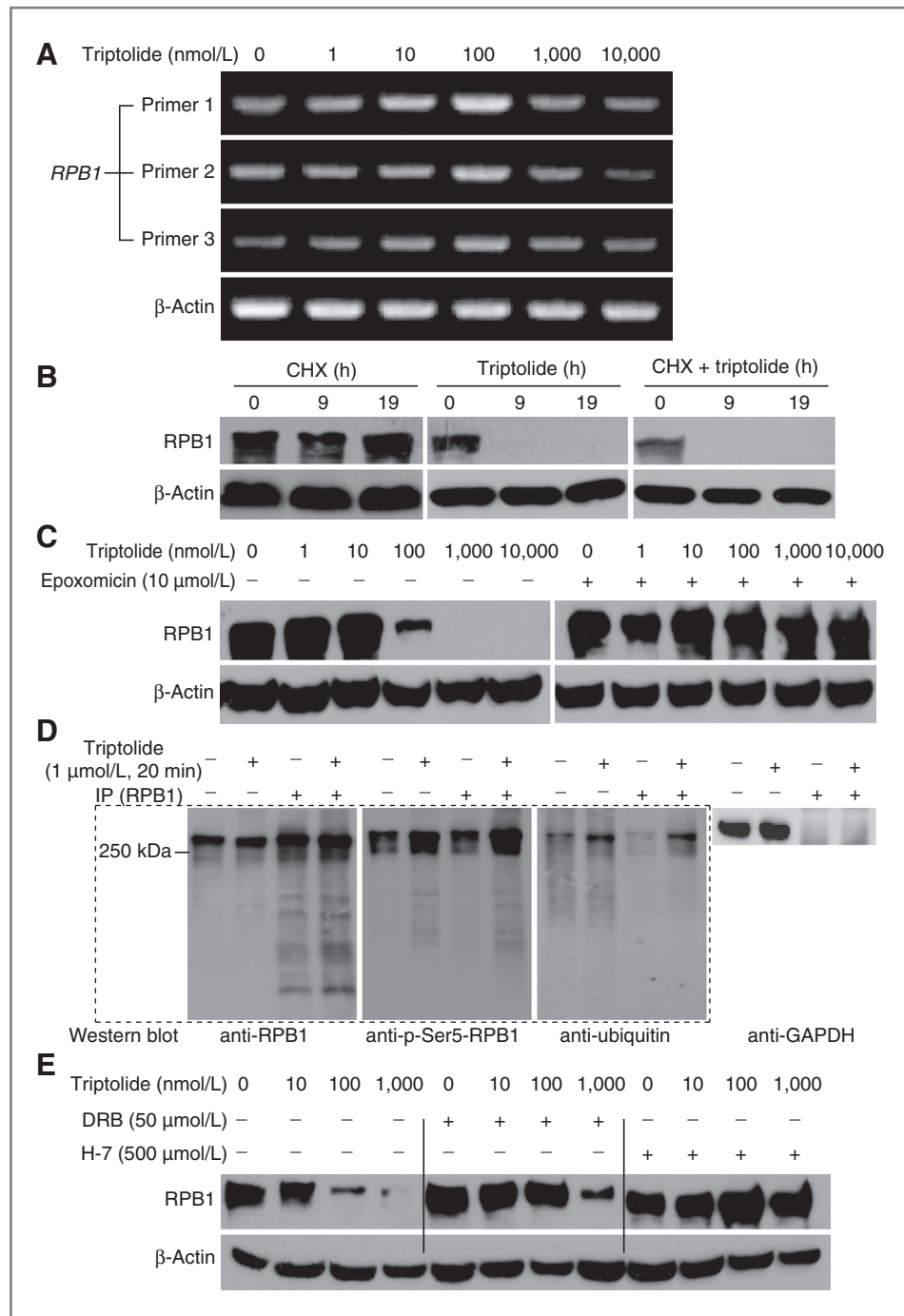
almost equally (3, 25). Our results revealed that at low concentrations ($\leq$100 nmol/L) of triptolide, DRB completely prevented the Rpb1 degradation, but at a high concentration (1,000 nmol/L) of triptolide, DRB rescued Rpb1 just partially. In contrast, H7 totally prevented the Rpb1 decrease triggered by triptolide at all tested concentrations (10–1,000 nmol/L; Fig. 2E). Together with the Rpb1 phosphorylation at Ser-5 rather than at Ser-2 induced by triptolide, the effects of both DRB and H7 suggest that triptolide could selectively cause the activation of Cdk7, which is likely to mediate the triptolide-induced Rpb1 degradation.

Triptolide-induced block of RNAPII at promoters precedes chromatin-bound enzyme reduction

The above results show a potential correlation of cellular RNAPII decrease with the phosphorylation at Ser-5 and ubiquitination of Rpb1 and its proteasome-dependent degradation induced by triptolide. However, we did not know whether

these effects of the drug indeed affected the engagement of RNAPII in transcription. Ser-5–hyperphosphorylated RNAPII is known to be mainly located near gene promoter regions (13). Therefore, we first conducted ChIP assays by using an antibody against the N-terminal domain of RNAPII in the PC-3 cells treated with triptolide to see whether the chromatin-bound RNAPII at the promoter regions of transcribed genes reduces. The data revealed that 2-hour treatments with 1 μmol/L triptolide resulted in a depletion of more than 90% of the RNAPII bound to the promoters of transcribed *myc*, *hif-1α*, *vegf*, and *polr2a* genes (Fig. 3A). Time course experiments with a lower drug concentration (100 nmol/L) further showed the significant decrease of RNAPII at those promoters after 3 hours (Fig. 3B). Interestingly, 100 nmol/L triptolide did not reduce, and even increased, in some cases the RNAPII density at the tested promoters at 2 hours (Fig. 3B). These results indicate that triptolide can trigger a reduction of the RNAPII density at those class II gene promoters in a concentration- and time-dependent manner, in agreement with the RNAPII depletion detected in cellular extracts (Fig. 1).

In addition to protein degradation, reduction of the RNAPII density at promoters could also result from an increased escape of RNAPII from promoter–proximal pausing sites as in the case of camptothecin (22, 26). To exclude this possibility, we conducted ChIP assays to detect the distribution of RNAPII at different sites along transcribed genes at different time points in PC-3 cells treated with 100 nmol/L triptolide. Triptolide induced a reduction of RNAPII at both promoters and exons in all the tested genes (Fig. 4). However, the reduction kinetics was significantly different at the promoters and the exons of those genes. At all the tested promoters, the decrease of the RNAPII density was not detectable at 1- and 2-hour time points though apparent after the 3-hour treatment; in contrast, the levels of RNAPII bound to the corresponding exons were markedly reduced at all the examined time points (1–4 hours; Fig. 4). Interestingly, we detected a significant increase of RNAPII at *c-myc* promoter after 1 hour of treatment, whereas a consistent polymerase decrease was found at the gene exons (Fig. 4A). Similar results were found at the other 2 genes, *vegf* and *hif-1α*, at the promoters of which RNAPII density was substantially unaltered after 1 to 2 hours of treatments, whereas it was consistently decreased at exons. After longer drug treatments, RNAPII was reduced also at promoter regions (Fig. 4).
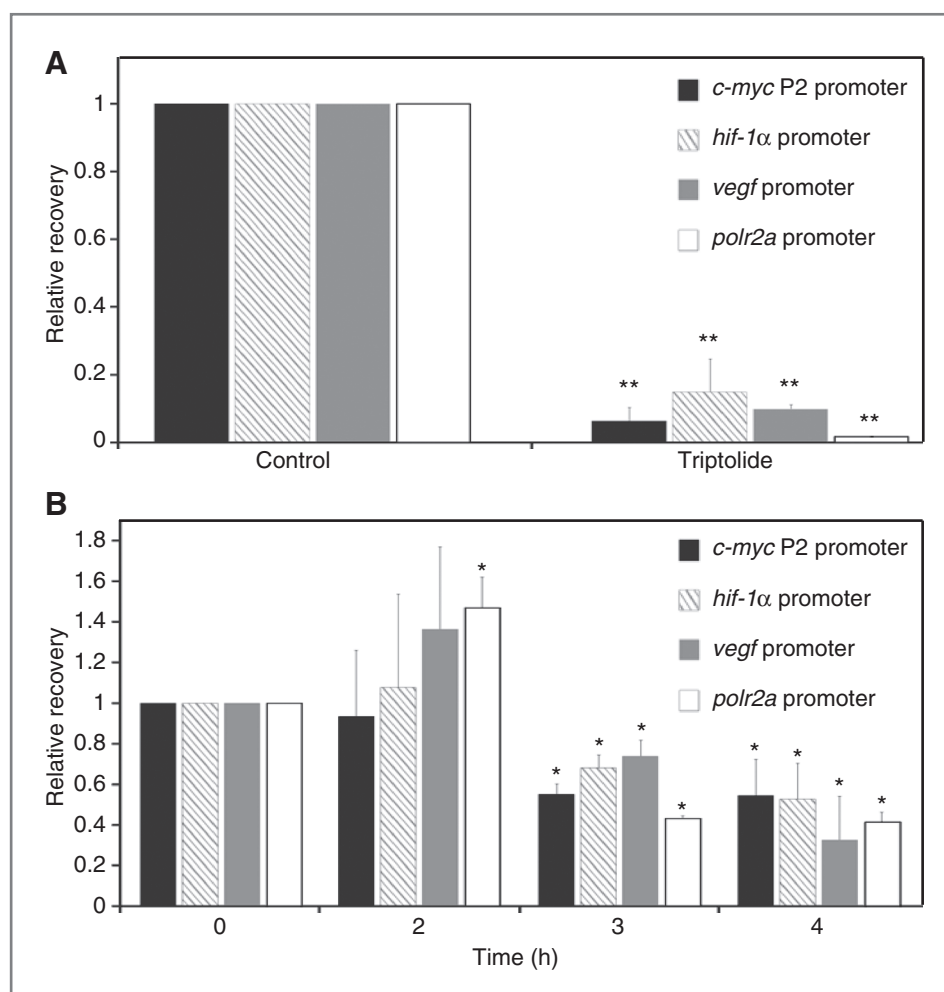
Figure 3. Triptolide induced reduction of the RNAPII bound to class II promoters. PC-3 cells were treated with 1 μmol/L triptolide for 2 hours (A) or with 100 nmol/L triptolide for the indicated time (B). The levels of the RNAPII bound to the indicated gene promoters were determined by ChIP with an antibody against the N-terminal domain of RNAPII. Values were normalized to the untreated control cells and expressed as mean ± SE of 9 determinations from 3 independent experiments (A) and of 6 determinations from 2 independent experiments (B). Mean relative recovery with preimmune IgG in all tested regions was 0.09 to 0.12. Mean relative recovery with a specific antibody at the α-satellite DNA region was 0.06. Statistically significant reduction was determined with the Student *t* test and indicated with **, *P* < 0.01 and *, *P* < 0.05.

Figure 4. A block of RNAPII at promoters precedes triptolide-induced RNAPII reduction. PC-3 cells were treated with 100 nmol/L triptolide for the indicated time. Chromatin-bound RNAPII was determined by ChIP with an antibody against the N-terminal domain of RNAPII. Values were normalized to the untreated control cells, and expressed as mean ± SD of 6 determinations from 3 independent experiments. Mean relative recovery with preimmune IgG was 0.06, and mean recovery with the specific antibody at the α-satellite DNA region was 0.08. The levels of chromatin-bound RNAPII were shown at different regions along *c-myc* (A), *vegf* (B), and *hif-1α* (C) genes. *P* values were calculated with the Student *t* test and indicated with **, *P* < 0.01 and *, *P* < 0.05.
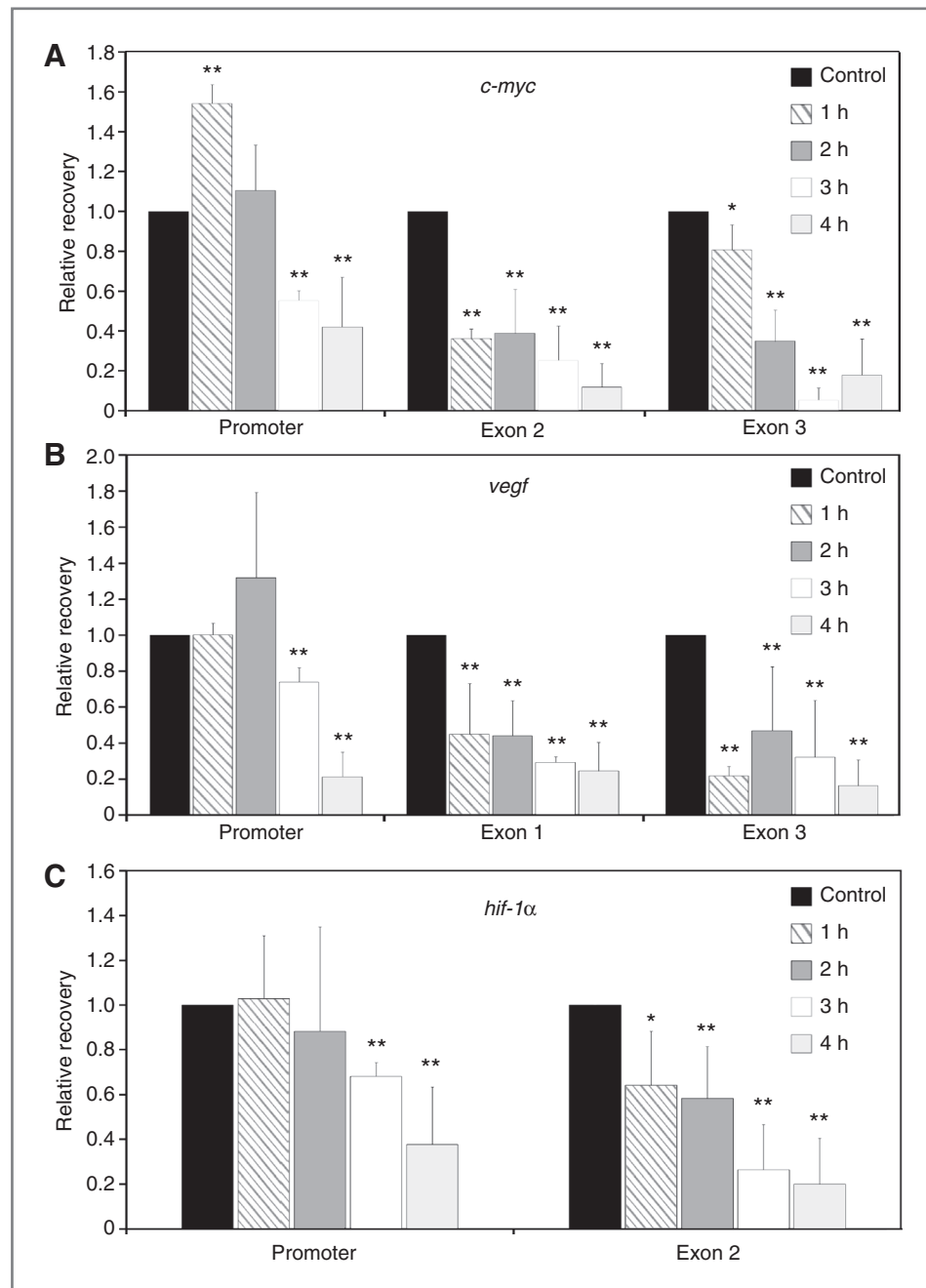
Thus, our present findings show that triptolide can first induce a block of RNAPII at the promoters of active genes. Moreover, chromatin-bound RNAPII can then be degraded likely due to the hyperphosphorylation at Ser-5. The degradation of chromatin-bound RNAPII can likely explain the reduction of cellular mRNA levels after triptolide treatments as reported previously (2, 4, 6).

Cdk7 mediates the triptolide-induced RNAPII degradation

To show the role of Cdk7 and Ser-5 hyperphosphorylation in the degradation of total and chromatin-bound RNAPII, we used specific siRNA to interfere with the expression of Cdk7 in PC-3 cells. Western blotting analyses on cellular protein extracts showed that downregulation of Cdk7 with its siRNA prevented the hyperphosphorylation of Rpb1 at its Ser-5 and its subsequent degradation induced by triptolide (Fig. 5A). Similarly, Cdk7 siRNA also apparently protected the RNAPII bound to the promoters of transcribed *myc*, *hif-1α*, *vegf*, and *polr2a* genes from degradation (Fig. 5B). The results show that the Cdk7-dependent phosphorylation of Rpb1 at Ser-5 of its CDT is required for triptolide-triggered degradation of both the total RNAPII and the RNAPII bound to the promoters of the transcribed genes.
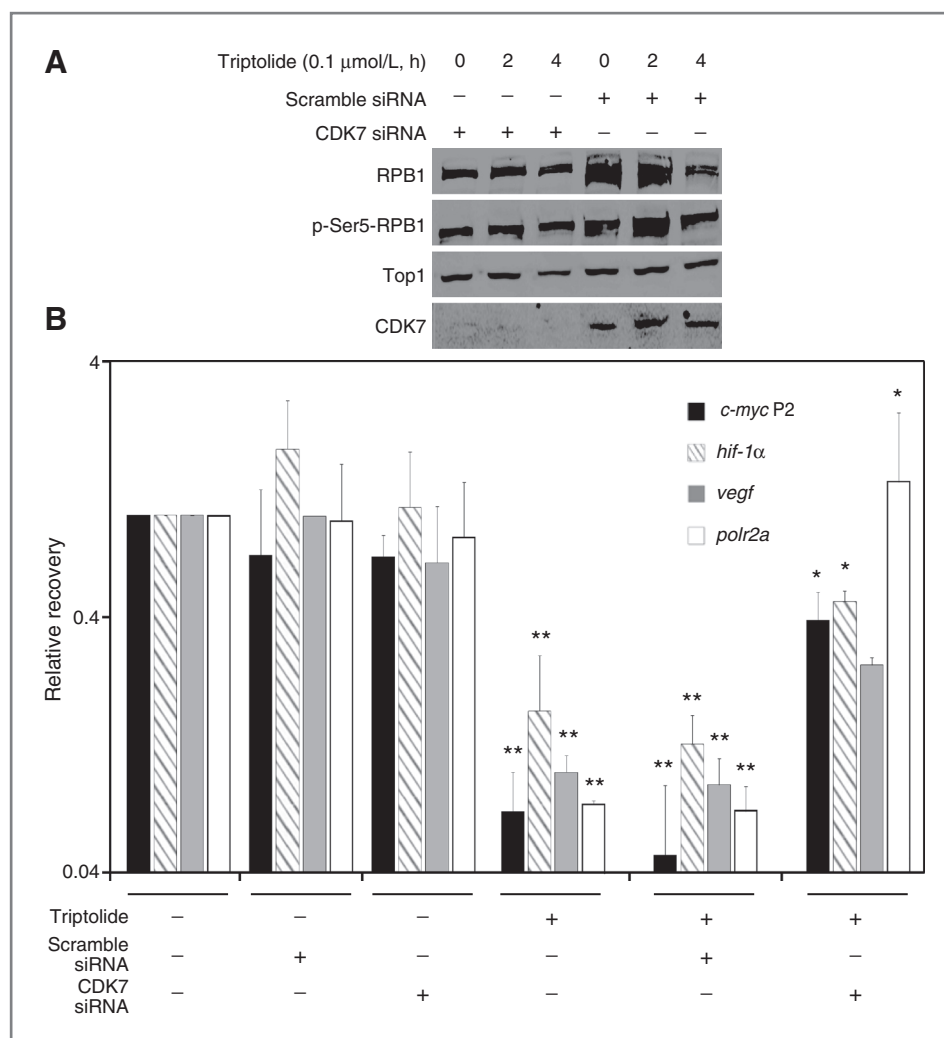
Figure 5. Cdk7 downregulation reverses the triptolide-induced Rpb1 degradation and Ser-5 hyperphosphorylation. PC-3 cells were transfected with scramble or CDK7-specific siRNA (20 nmol/L). After 72 hours, cells were treated with triptolide for the indicated time. Triptolide was used at 0.1 μmol/L (A) and 1 μmol/L (B). Nuclear proteins (20 μg) were analyzed by Western blotting (A) to assess the levels of Rpb1, Cdk7, and phosphorylated Rpb1 at Ser-5. DNA topoisomerase I (Top1) was used as the loading control. The RNAPII bound to the promoters was determined by ChIP (B) with an antibody against the N-terminal domain of RNAPII. Values were normalized to the untreated control cells and expressed as mean ± SE of 6 determinations from 2 independent experiments. Mean relative recovery with preimmune IgG was 0.13. Mean relative recovery with the specific antibody at the α-satellite DNA region was 0.004. P values were calculated with the Student t test and indicated with **, $P < 0.01$ and *, $P < 0.05$.

Discussion

The transcription factor TFIIH consists of 10 subunits including XPB and Cdk7 (27). TFIIH is a basal factor functioning at the stages of transcription initiation, promoter escape, and early elongation. XPB with its DNA-dependent ATPase activity opens the DNA around the transcription start site and Cdk7 by its kinase activity phosphorylates Rpb1 at Ser-5 of the CTD. Both are required for transcription initiation (27, 28). Previously, triptolide was reported to covalently bind to XPB and inhibit its ATPase activity, resulting in the inhibition of RNAPII-driven transcription (4). Here, we further indicate that Cdk7 mediates triptolide-induced Rpb1 degradation via increasing its phosphorylation at Ser-5.

In this study, we showed that triptolide-induced cellular RNAPII (specifically, its largest subunit Rpb1) decrease in different cells and importantly, this ability of the drug was proportionally correlated with its tumor cell-killing activity (Fig. 1D). Moreover, the results indicate that RNAPII is not reduced at promoters up to 2 hours of treatments (rather it is increased in the case of *c-myc*), whereas it is reduced at all the exons examined for the same time periods. Thus, we can

suggest that RNAPII block at promoters is an early event induced by triptolide (Figs. 3–5).

These effects confer apparent biologic significance to further exploration on the potential molecular mechanism. Triptolide was shown to lead to the Rpb1 hyperphosphorylation at Ser-5 rather than at Ser-2 and the increased Rpb1 ubiquitination; proteasome inhibitors and Cdk7 inhibitors were revealed to prevent the triptolide-induced Rpb1 reduction (Figs. 1 and 2). These data indicate a strong possibility that triptolide triggers a Cdk7-mediated proteasome-executed Rpb1 degradation mechanism. Cdk7 downregulation by its siRNA interference rescued the decrease of the cellular and chromatin-bound Rpb1 induced by triptolide, therefore validating this possibility.

Comparison of our results with the previous related reports (2, 4, 6) indicates that the triptolide induced proteasome-dependent Rpb1 degradation and the correlation with its inhibition of RNAPII-mediated transcription are clear-cut. However, there is one apparently inconsistent but important point, that is whether, where, and how triptolide causes Rpb1 hyperphosphorylation. Titov and colleagues (4) did not observe

that triptolide had any effect on the phosphorylation of Rpb1. According to our data, triptolide induced a biphasic change of first rising and then declining in the levels of phosphorylation at the CTD (Fig. 1B and C). Therefore, it is possible that their experimental conditions (200 nmol/L triptolide, 1 and 4 hours, in A549 cells) are not the optimal ones for this purpose. Wang and colleagues (6) indeed detected the Rpb1 hyperphosphorylation elicited by the drug. However, they used an antibody against Rpb1 but not antibodies against Rpb1 phosphorylated at specific sites. Therefore, their results did not directly reveal the site where the hyperphosphorylation of Rpb1 took place, although they used DRB and H7, both of which are able to inhibit more than one kinases including CDK7 and CDK9 (3, 25), to indirectly infer that Ser-2 of the CTD could be the site. In contrast, our study used specific antibodies, respectively, against Rpb1 phosphorylated at Ser-2 and at Ser-5 to clearly show that the site is Ser-5 rather than Ser-2. At the same time, our data also showed the biphasic kinetics of triptolide-induced Rpb1 hyperphosphorylation at Ser-5 (Figs. 1B and C and 2D). Wang and colleagues further used CDK9 siRNA and cyclin T1 siRNA in their report. However, it is hard to conclude that these siRNAs indeed prevented the triptolide-induced Rpb1 hyperphosphorylation because of the poor quality of Rpb1 Western blotting images (6). In this study, our data revealed that Cdk7 siRNA prevented the Ser-5 hyperphosphorylation of Rpb1 caused by triptolide (Fig. 5A). Thus, our results clearly indicate that triptolide gives rise to the Cdk7 activation that mediates the Rpb1 hyperphosphorylation at Ser-5. However, we cannot fully exclude that Cdk9 can contribute to the hyperphosphorylation of Rpb1 as it has also been shown to phosphorylate the CTD at Ser-5 of Rpb1 in vitro (29).

Our study further indicates that the pharmacologic inhibitors and siRNA of Cdk7 can reverse the proteasome-dependent degradation of the cellular and chromatin-bound Rpb1 induced by triptolide (Figs. 2E and 5). This finding shows that the CTD plays a role in maintaining Rpb1 stability, and can further help to clarify the other controversial issue of whether phosphorylation of the CTD is correlated with its ubiquitination and Rpb1 degradation (8, 10, 16, 17). In addition to the standard heptapeptide consensus sequence of $Y_1S_2P_3T_4S_5P_6S_7$, in fact, there are several heptapeptide variants, including 5 repeats of $Y_1S_2P_3T_4S_5P_6K_7$, 2 repeats of $Y_1T_2P_3T_4S_5P_6K_7$, and 1 sequence of $Y_1S_2P_3T_4T_5P_6K_7$ (Fig. 6, top; http://www.ncbi.nlm.nih.gov/nuccore/306482654). This is interesting because there is not only a lysine residue at the 7 position but also a serine residue at the 5 position of those sequences except the last one. These sites are likely to be the potential targets of the E3 ligase for polyubiquitination (Lys7; ref. 30) and Cdk7 for phosphorylation (Ser-5). TFIIH concomitantly possesses these 2 activities of the kinase for Ser-5 phosphorylation and the E3 ligase attributing to its subunits of Cdk7 and p44, respectively (30, 31). Moreover, triptolide has been shown to bind to the XPB subunit, but not to other subunits of TFIIH (4). Together with our data in this study, we therefore propose a possible mechanism by which triptolide causes Rpb1 degradation and transcriptional inhibition (Fig. 6, bottom). The binding of triptolide to XPB might, via unknown mechanism(s) (probably via the change in the conformation of the complex as the XPB homolog structure implies a major conformational change upon ATP binding; ref. 32), activate Cdk7 and p44. The activated Cdk7 could lead to the Ser-5 phosphorylation at the standard heptapeptide consensus sequence of Y1S2P3T4S5P6S7 and the heptapeptide variants of Y1S/T2P3T4S5P6K7. The former might cause RNAPII stalling and, the latter might facilitate the polyubiquitination mediated by the activated p44, which could be potentiated by the RNAPII stalling. The ubiquitination of Rpb1 results in its proteasome-dependent degradation. As we did not determine the acceptor sites of the ubiquitin chain in Rbp1 in response to triptolide treatments, we cannot exclude the possibility that Rbp1 is ubiquitinated outside from the CTD.

Figure 6. A proposed mode of action of triptolide. Top, the standard heptapeptide consensus sequence of $Y_1S_2P_3T_4S_5P_6S_7$ and the heptapeptide variants including $Y_1S_2P_3T_4S_5P_6K_7$, $Y_1T_2P_3T_4S_5P_6K_7$ and $Y_1S_2P_3T_4T_5P_6K_7$ in the CTD of Rpb1 beginning at amino acid residue 1615 (adapted from http://www.ncbi.nlm.nih.gov/nuccore/306482654). The shading between amino acid residues 1615 and 1804 is to highlight the standard heptapeptide consensus sequence of $Y_1S_2P_3T_4S_5P_6S_7$. The shading between amino acid residues 1832 and 1937 is to highlight 8 heptapeptide variants, including 5 repeats of $Y_1S_2P_3T_4S_5P_6K_7$, 2 repeats of $Y_1T_2P_3T_4S_5P_6K_7$, and 1 sequence of $Y_1S_2P_3T_4T_5P_6K_7$. All these variants contain a lysine residue at their seventh site. Bottom, a proposed mode of action of triptolide.
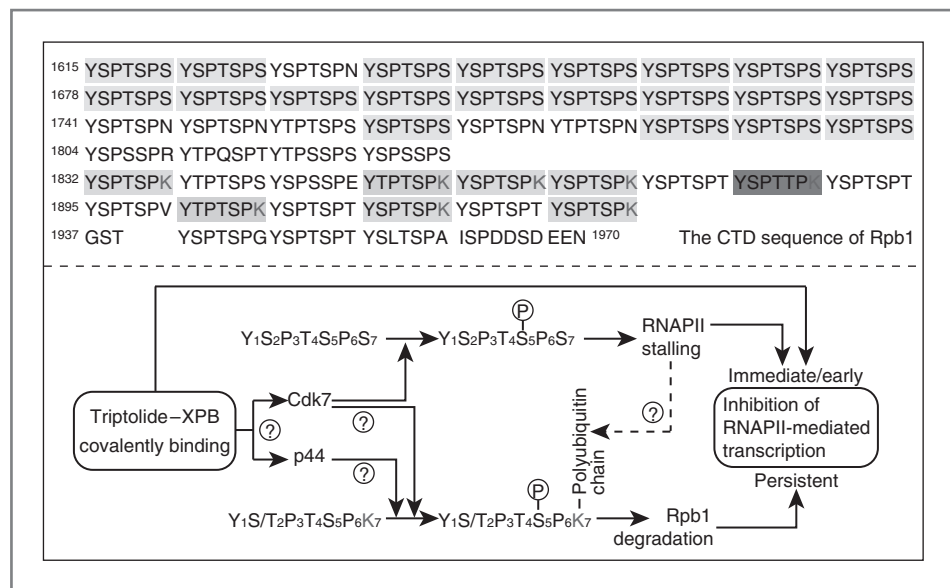
It could be possible that the binding of triptolide to XPB and the RNAPII stalling elicit immediate or early transcriptional inhibition, and the subsequent persistent inhibition results from the decrease of RNAPII due to the Rpb1 degradation.

The proposed mechanism could reasonably unify all the Ser-5 hyperphosphorylation, ubiquitination-mediated Rpb1 degradation, and RNAPII-driven transcriptional inhibition induced by triptolide into 1 primary target XPB and 1 related complex TFIIH. Our findings show that triptolide is a transcriptional inhibitor unique and distinct from all other known transcriptional inhibitors including DRB, flavopiridol, actinomycin D, and α-amanitin (3, 4, 33). Thus, triptolide could serve as an exquisite tool to further investigate the regulation and function of TFIIH, and act as a model compound for anticancer/other drug development specifically targeting the transcription factor TFIIH.

However, further work is required to clarify a number of important questions about this possible mechanism. In particular, does the binding to XPB inhibit polymerase escape from promoters, or does the hyperactivation of Cdk7 induce RNAPII stalling? Do phosphorylation and ubiquitination indeed occur at Ser-5 and Lys7 in the heptapeptide variants of $Y_1S/T_2P_3T_4S_5P_6K_7$, respectively? Does the subunit p44 act as the E3 ligase for ubiquitination at Lys7? What roles do the other subunits play in regulating the enzymatic activities of XPB, Cdk7, and p44? How does the binding of triptolide to XPB affect other subunits such as Cdk7 and p44? The answers to those questions will help not only to greatly expand the knowledge about triptolide but also to more fully understand the regulation and function of TFIIH.

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