Alleviation of PC4-mediated Transcriptional Repression by the ERCC3 Helicase Activity of General Transcription Factor TFIIH*

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Positive cofactor 4 (PC4), originally identified as a transcriptional coactivator, possesses the ability to suppress promoter-driven as well as nonspecific transcription via its DNA binding activity. Previous studies showed that the repressive activity of PC4 on promoter-driven transcription is alleviated by transcription factor TFIIH, possibly through one of its enzymatic activities. Using recombinant TFIIH, we have analyzed the role of TFIIH for alleviating PC4-mediated transcriptional repression and determined that the excision repair cross-complementing (ERCC3) helicase activity of TFIIH is the enzymatic activity that alleviates PC4-mediated repression via β-γ bond hydrolysis of ATP. In addition, the alleviation does not require either ERCC2 helicase or cyclin-dependent kinase 7 kinase activity. We also showed that, as complexed within TFIIH, the cyclin-dependent kinase 7 kinase does not possess the activity to phosphorylate PC4. Thus, TFIIH appears to protect promoters from PC4-mediated repression by relieving the topological constraint imposed by PC4 through the ERCC3 helicase activity rather than by reducing the repressive activity of PC4 via its phosphorylation.

Positive cofactor 4 (PC4)† was originally identified in the upstream-factor stimulatory activity that augments activator-dependent transcription in vitro (1, 2). PC4 stimulates transcription in vitro with diverse kinds of activators, including VP16 (3, 4), thyroid hormone receptor (5), octamer transcription factor-1 (6), and BRCA-1 (7), presumably by facilitating assembly of the preinitiation complex through bridging between activators and the general transcriptional machinery (4, 8). Studies on the interaction of PC4 with activators and TFIIA, as well as in vitro functional analyses, suggest that interaction between TFIIA and PC4 plays a pivotal role for facilitating the preinitiation complex (PIC) assembly (3, 4). Further studies also demonstrated the importance of PC4 for transcriptional activation by AP-2 (9) and HIV transactivator Tat (10) in vivo. In addition, a yeast homologue of PC4, SUB1/TSF1 (11, 12), which is essential for viability in the presence of TFIIH mutations (12), was shown to function as a coactivator for GCN4 and HAP proteins. The N-terminal region of PC4 contains a serine-rich portion termed the SEAC domain, which exhibits similarity to viral immediate-early proteins (3). Phosphorylation of the serine residues in the SEAC domain negatively regulates the coactivator activity of PC4 (3, 13) possibly by a conformational change.

In addition to the role as coactivator, PC4 was subsequently shown to repress promoter-driven transcription as well as nonspecific transcription in vitro (14, 15). The analyses of PC4 mutants demonstrated that the repressive activity is a separate function from the coactivator activity (14); therefore, the repressive activity of PC4 may play an as yet unknown function in regulating transcription in vivo. In fact, the primary function of PC4 in vivo could possibly be to repress transcription rather than to enhance transcription because phosphorylated PC4, which is inactive as a coactivator but retains repressive activity, is the predominant form (∼95%) within the cells (13). Transcriptional repression by PC4 correlates with the single-stranded (ss) DNA binding activity present in its C-terminal region, which shows preferential binding to melted double-stranded (ds) DNA and to heteroduplex DNA (14). The structural studies show that PC4 forms a homodimer via its C-terminal region that contains four-stranded β-sheets rich in positively charged and aromatic residues involved directly in binding to ssDNA (16, 17). Interestingly, in contrast to its coactivator activity, the ssDNA binding activity of PC4 is augmented by phosphorylation of its N-terminal region (8). Further studies indicate that PC4-mediated repression of specific transcription from promoters is alleviated by TFIIH, possibly through its enzymatic activities that require β-γ hydrolysis of ATP (14, 15). However, the identity of the enzymatic activity responsible for the alleviation as well as the mechanism by which TFIIH alleviates PC4-mediated repression remains unknown.

Here we used the recombinant TFIIH mutants that lack one of the enzymatic activities (cdk7 kinase, ERCC2 helicase, or ERCC3 helicase) (18) and examined the mechanism by which TFIIH counteracts the repressive effect of PC4. We have found that TFIIH counteracts PC4-mediated repression via ERCC3 helicase activity and that neither ERCC2 helicase nor cdk7 kinase activity is required for alleviating the repression, an observation further supported by the fact that TFIIH does not phosphorylate PC4. Our results suggest that PC4 and the ERCC3 helicase activity of TFIIH may act together to increase the specificity of transcription and also to provide more intricate regulation of transcription.

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§ The abbreviations used are: PC4, positive cofactor 4; ERCC, excision repair cross-complementing; cdk, cyclin-dependent kinase; PIC, preinitiation complex; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; RNAPII, RNA polymerase II; TBP, TATA box-binding protein; CTD, carboxy-terminal domain; Ni-NTA, nickel-nitri lodiacetic acid; TF, transcription factor; nt, nucleotide; HIV, human immuno deficiency virus.

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EXPERIMENTAL PROCEDURES

Purification of Transcription Factors—PC4 was expressed in Escherichia coli, BL21(DE3)pLysS, harboring the plasmid pET11c-PC4, and the extract was prepared by sonication in buffer A (20 mM Hepes-KOH, pH 7.9, 10% glycerol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol containing 100 mM KCl). The extract was applied onto a Hitrap SP column, and the bound proteins were eluted with a 5-column volume of a linear gradient of 0.1–0.6 M KCl. The eluted fractions were diluted to adjust the conductivity to that of 0.1 M KCl and then loaded onto a Hitrap heparin column. The bound proteins were eluted with a 5-column volume of linear gradient of 0.1–0.6 M KCl. RNA polymerase II (RNAPII), TFIIH, TFIIIE, and TFIIF, and FLAG-tagged TBP (f:TBP) were prepared essentially as described (19).

Preparation of Recombinant TFIIH—Recombinant TFIIH and its mutants were reconstituted in High Five cells using three baculoviruses, each of which expresses three subunits of TFIIH (18, 19). The purification of TFIIH was done essentially as described (19) except that TALON™ metal affinity resin (Clontech) was used in place of Ni-nitrotriacetic acid (NTA) superflow (Qiagen). The amount of each TFIIH, whose cdk7 subunit is C-terminal-tagged with a FLAG epitope, was adjusted by using silver-stained gels as well as quantitative immunoblots with anti-FLAG M2 antibody.

In Vitro Transcription—In vitro transcription reactions were carried out in a 25-μl reaction containing 12 mM Hepes-KOH, pH 7.9, 6% glycerol, 60 mM KCl, 0.6 mM EDTA, 8 mM MgCl₂, 5 mM dithiothreitol, 20 units of RNase inhibitor (TaKaRa), 0.2 mM ATP, 0.2 mM UTP, 0.1 mM 3′-O-methyl GTP, 12.5 μM CTP, 10 μM of (α-32P)CTP, 20 ng TFIIA, 10 ng TFIIB, 4 ng TFIIE, 10 ng TFIIH, 20 ng recombinant TFIIH, 100 ng RNAPII, and the indicated amount of PC4. All the transcription reactions contained negatively supercoiled pML535 (100 ng) as a template. The reactions were performed at 30°C for 1 h, stopped by the addition of 20 mM EDTA, 0.2% SDS, and 5 μg of proteinase K, and further incubated at 37°C for 1 h. After phenol/chloroform extraction and ethanol precipitation, the transcripts were analyzed by electrophoresis on a 5% denaturing polyacrylamide gel, followed by autoradiography.

Kinase Assays—Phosphorylation of GST-CTD (carboxyl-terminal domain) and PC4 by TFIIH was performed essentially as described (19). Where indicated, casein kinase II (New England Biolabs) was used in place of TFIIH as indicated.

RESULTS

Requirement of β–γ Bond Hydrolysis of ATP for the Alleviation of PC4-mediated Repression by TFIIH—To investigate the functional relationship between TFIIH and PC4, we prepared recombinant TFIIH reconstituted in the insect cells infected with three baculoviruses that expressed TFIIH subunits (18, 19). In vitro transcription assays were performed with recombinant TBP, TFIIH, TFIIE, TFIIF, and TFIIH together with RNAPII purified from HeLa cells (Fig. 1A), using a linearized pML535C2AT template that contained the adenovirus major late promoter fused with a 380-bp G-less cassette (19). The specific 390-nt transcript was observed only in the presence of all factors. No transcription was observed when one of the factors was omitted from the reaction, indicating that there was no cross-contamination among the factors (Fig. 1B).

We next tested whether recombinant TFIIH could alleviate transcriptional repression by PC4. As shown in Fig. 2A, even in the absence of TFIIH, the negatively supercoiled template allowed production of the specific 390-nt transcript (lane 1), which was suppressed to less than 5% by the addition of PC4 (lane 2). Adding the increasing amounts of TFIIH, however, gradually restored the levels of transcription (lanes 3–6) to 40–60% of those seen in the absence of both TFIIH and PC4 (lane 1), indicating that recombinant TFIIH can reverse the repressive effect of PC4 in a dose-dependent manner as does natural TFIIH (14, 15).

Using the highly purified reconstituted system, we then tested the requirement for β–γ bond hydrolysis by substituting ATP with adenylyl-imidodiphosphate (AMP-PNP) and adenosine-5′-O-(thiotriphosphate) (ATP-γS), both of which can be incorporated into growing RNA chains during transcription but cannot be hydrolyzed at the β–γ bond. When ATP was replaced by non-hydrolyzable AMP-PNP or ATP-γS in the transcription reactions containing both PC4 and TFIIH, virtually no transcription was observed (Fig. 2B, lanes 4 and 6), indicating that β–γ bond hydrolysis of ATP was absolutely required for counteracting PC4-mediated repression. Transcription was restored, however, when AMP-PNP and ATP-γS were further supplemented with dATP (Fig. 2B, lanes 5 and 7), which could provide β–γ bond hydrolysis. These results show the require-
The requirement of ERCC3 helicase activity for alleviating PC4-mediated repression requires ERCC3 helicase activity. The transcription reactions contained TBP, TFIIH, TFIIE, TFIIF, PC4, and RNAPII, together with 5, 10, 20, and 40 ng of wild-type TFIIH (lanes 3–6), K41A (lanes 7–10), K346A (lanes 11–14), or K48A (lanes 15–18) as indicated.

TFIIH Does Not Phosphorylate PC4—Because the previous result showed that PC4 is released from the template upon phosphorylation by TFIIH (21), the dispensability of the cdk7 kinase for PC4-mediated repression was somewhat unexpected. Furthermore, lack of any consensus phosphorylation site for cdks (S/TPPXR/K) in PC4 prompted us to re-address whether TFIIH is indeed able to phosphorylate PC4 in vitro as previously reported (15, 21). As shown in Fig. 4A, wild-type TFIIH, K48A, and K346A phosphorylated CTD efficiently but K41A did not phosphorylate CTD, indicating that the substitution of lysine with alanine at the 41st residue of cdk7 eliminated the kinase activity to an undetectable level. Phosphorylation of CTD by TFIIH produced the hypophosphorylated form as well as the hyperphosphorylated form that showed a slower migration on the SDS gel (Fig. 4A). Casein kinase II also phosphorylated CTD, although phosphorylation did not shift the migration of GST-CTD (Fig. 4A, lanes 1 and 2).

We next tested whether casein kinase II and the same set of TFIIH mutants could phosphorylate PC4. Casein kinase II efficiently phosphorylated PC4 as previously reported (3, 13) and altered PC4 from the faster migrating form (~15 kDa) to the slower migrating form (~20 kDa) (Fig. 4B, bottom panel, lanes 1 and 2). In contrast, wild-type TFIIH, K346A, and K48A, all of which retain cdk7 kinase activity (Fig. 4A, lanes 4, 6, and 7), did not phosphorylate PC4 (Fig. 4B, lanes 4, 6, and 7). The low levels of PC4 labeling observed on a longer exposure of the gel (Fig. 4B, middle panel, lanes 3–7) is not because of the TFIIH kinase activity because the TFIIH mutant K41A, which lacks the kinase activity (Fig. 4A, lane 5), showed the same degree of labeling as wild-type TFIIH. Our results demonstrate that TFIIH does not phosphorylate PC4 and argues against the involvement of PC4 phosphorylation by TFIIH for alleviating PC4-mediated repression of transcription.

Quantitative Analysis of PC4-mediated Repression in Absence of the ERCC3 Helicase Activity—The in vivo concentration of PC4 is estimated to be ~1 μM in HeLa cells, and ~95% of PC4 is phosphorylated in vivo, presumably by casein kinase II (3, 13, 14). Therefore, we tested whether phosphorylated and non-phosphorylated PC4 can distinguish the presence and ab-
The relative levels of transcription were shown. The level of transcription in the absence of PC4 was arbitrarily defined as 1.0. Transcription reactions contained TBP, TFIIH, TFIIE, TFIIF, and RNAPII in the presence of either wild-type TFIIH (WT) or the ERCC3-deficient TFIIH mutant (K346A), together with indicated amounts of either non-phosphorylated or phosphorylated PC4. The values indicate the level of transcription in the presence of wild-type TFIIH and non-phosphorylated PC4 (filled squares, solid line), wild-type TFIIH and phosphorylated PC4 (open squares, dotted line), K346A and non-phosphorylated PC4 (filled triangles, solid line), and K346A and phosphorylated PC4 (open triangles, dotted line).

Our results show that the ERCC3 helicase activity of TFIIH counteracts PC4-mediated transcriptional repression and that neither the ERCC2 helicase nor the cdk7 kinase has any role in this process. The fact that the ERCC3 helicase, but not the cdk7 kinase, of TFIIH relieves PC4-mediated repression provides a clue as to the mechanism by which TFIIH and PC4 act antagonistically to regulate transcription. Negatively supercoiled templates allow specific transcription by RNAPII in the absence of TFIIH and ATP in vitro (22, 23), presumably by the transfer of free energy stored on the negatively supercoiled templates (24–26). This transfer of free energy appears to be constrained by PC4, because the property of negatively supercoiled DNA templates bound by PC4 is similar to that of linear DNA templates with regard to the absolute requirement of TFIIH and ATP for specific promoter-driven transcription (22, 23). This effect of PC4 transmitted indirectly through DNA to the general transcriptional machinery is consistent with the functional antagonism between TFIIH and PC4 that does not involve the mutual exclusion of TFIIH and PC4 on the promoter region (21). Our results, however, rule out the possibility that the cdk7 kinase of TFIIH phosphorylates PC4 (15, 21) and facilitates its release from the promoter region.

In light of our study as well as a previous study (14), we propose two possible mechanisms by which PC4 represses promoter-independent transcription: i.e. “direct” and “indirect” mechanisms. In the direct mechanism, PC4 binds to ssDNA regions with its ssDNA binding ability, competing directly with RNAPII, and thus physically displaces RNAPII from ssDNA regions (Fig. 6A). By contrast, in the indirect mechanism PC4 binds dsDNA regions via its dsDNA binding ability and renders DNA more “rigid” so that the free energy stored in negative superhelicity (24–26) will not generate transiently melted ssDNA regions that permit RNAPII to initiate random transcription (Fig. 6B). It is conceivable that the indirect mechanism provides the primary protection against spurious transcription and the direct mechanism provides a backup. In this scenario, PC4 bound to ssDNA regions may also serve as a reservoir that can be recruited quickly to ssDNA regions where the possibility of spurious transcription is greater. In agreement with the recruitment of PC4 from dsDNA to ssDNA, PC4 binds to ssDNA more strongly than to dsDNA (14).

PC4-mediated repression of transcription from non-promoter regions as described above may facilitate the efficient allocation of the limiting amount of RNAPII in vivo (27, 28), which could be otherwise sequestered onto transiently melted ssDNA regions. In the living cells, DNA is predominantly negatively supercoiled and is also undergoing dynamic topological changes during DNA replication, transcription, and repair, possibly exposing melted ssDNA regions frequently. Spurious transcription from these melted ssDNA regions is likely to be suppressed mainly by phosphorylated PC4, which constitutes ~95% of PC4 in vivo (13), because phosphorylated PC4 can strongly suppress promoter-independent (and thus, general transcription factor-dependent) transcription from the melted DNA region in vitro (14).

PC4 may also play a role in preventing spurious transcription from promoters, which in vivo is likely to be negatively supercoiled and from which transcription could be potentially initiated in the absence of TFIIH. When the ERCC3 helicase of
TFIIH is active within the general transcriptional machinery, transcription is probably not repressed by PC4 in vivo (Fig. 6C) because the TFIIH ERCC3 helicase activity counters the repressive activity of phosphorylated PC4 at the physiological concentration (≈1 μM) (Fig. 5). Indeed, when PC4 is overexpressed in cells in the absence of the HIV transactivator, transcription from the HIV promoter is only marginally reduced or not reduced at all, depending upon the assay conditions (10). However, if the ERCC3 helicase activity of TFIIH is inhibited (Fig. 5), such as by negative regulator of activated transcription and by FBP interacting repressor (29, 30), phosphorylated PC4 may further reduce the low background transcription from promoters even at the physiological PC4 concentration (Fig. 6D). Because TFIIH appears to be sub-stoichiometric (20–30%) to other general transcription factors in vivo (27), a fraction of PIC might even lack TFIIH and could be repressed by PC4, though this possibility must be rigorously examined in vivo. In any event, regulation of promoter-dependent transcription with a high level of dynamic range in vivo is likely to be contingent upon the presence of PC4, because negatively supercoiled DNA in vivo may permit inadvertent transcription from promoters and could potentially reduce the dynamic range of transcriptional regulation.

Several lines of evidence suggest the importance of PC4 in regulating transcription in vivo. First, a yeast homolog of human PC4, SUB4, enhances transcriptional activation by the activators GCN5 and HAP4 (12), and though PC4 is not essential for viability, its deletion results in inositol auxotrophy, a phenotype observed in the mutations of transcriptional regulators such as SNF/SWI, SRB, and the CTD of RNA polymerase II (31–34). Second, PC4 enhances TAT-dependent transcription from the HIV promoter (10) and restores the reduced AP-2 activity in the ras-transformed cell lines by relieving AP-2 self-interference (9). Finally, PC4 may play a role as a tumor suppressor in lung and bladder cancers, because the loss of heterogeneity of the PC4 gene is often observed in these cancer cells (35, 36). These results demonstrate the importance of PC4 as a regulator of transcription and possibly as a tumor suppressor in vivo. Though the importance of PC4 in vivo has been mainly interpreted in the context of its coactivator activity, the predominance of the repressive form of PC4 in vivo (13) suggests that some of the observed effects may well be attributed to the reduced precision of transcriptional regulation caused by the loss of the repressive activity of PC4.

In conclusion, the repressive activity of PC4 may be essential for the intricate regulation of transcription in conjunction with the ERCC3 helicase of TFIIH. The repressive activity of PC4, and possibly of other ssDNA-binding proteins, may play an important but yet under-appreciated role for more elaborate and fine-tuned regulation of reactions involving DNA molecules.

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