Human PC4 Is a Substrate-specific Inhibitor of RNA Polymerase II Phosphorylation*

(Received for publication, December 15, 1999, and in revised form, January 6, 2000)

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The activity of cyclin-dependent protein kinases (cdks) is physiologically regulated by phosphorylation, association with the specific cyclin subunits, and repression by specific cdk inhibitors. All three physiological regulatory mechanisms are specific for one or more cdks, but none is known to be substrate specific. In contrast, synthetic cdk peptide inhibitors that specifically inhibit cdk phosphorylation of only some substrates, “aptamers,” have been described. Here, we show that PC4, a naturally occurring transcriptional coactivator, competitively inhibits cdk-1, -2, and -7-mediated phosphorylation of the largest subunit of RNA polymerase II (RNAPII), but it does not inhibit phosphorylation of other substrates of the same kinases. Interestingly, the phosphorylation of PC4 is devoid of kinase inhibitory activity. We also show that wild-type PC4 but not the kinase inhibitory-deficient mutant of PC4 represses transcription in vivo. Our results point to a novel role for PC4 as a specific inhibitor of RNAPII phosphorylation.

Phosphorylation of RNAPII plays an important role in transcription initiation and elongation (reviewed in Ref. 1). A number of cyclin-dependent protein kinases (cdks), including the cdk7 component of transcription factor IIB (TFIIB), are known to catalyze the phosphorylation of RNAPII (2–8). Because the activity of many cdks is regulated in vivo and in vitro by specific cdk inhibitors (9–16), we reasoned that specific cdk inhibitors might also exist that regulate phosphorylation of RNAPII. In our search for specific inhibitors of RNAPII phosphorylation, we discovered that the transcriptional coactivator PC4 (17, 18) has the ability to inhibit cdk7-mediated phosphorylation of RNAPII. PC4 was initially described as a DNA-binding protein that enhances the activator-dependent transcription of class II genes in vitro (17, 18). It was later shown that PC4 could also act as a repressor of basal transcription (19, 20). The DNA binding activity of PC4 allows the protein to bind to single-stranded and melted double-stranded DNA (21–24). PC4 has also been shown to be able to participate in DNA replication, substituting for other ssDNA-binding proteins in vitro (25). Here we show that PC4 inhibits the phosphorylation of the largest subunit of RNAPII by cdk7, cdk1, and cdk2. The inhibition of RNAPII phosphorylation by PC4 is specific, as phosphorylation of several other cdk substrates is not inhibited. This kinase inhibitory activity of PC4 is correlated with its transcriptional inhibitory activity in vivo.

MATERIALS AND METHODS

Inhibition of RNAPII Phosphorylation—Bacterially expressed PC4 was purified as described (18). TFIH, Cak, and RNAPII were purified as described (26). No PC4 or 37.5 or 75 ng of bacterially expressed PC4 were mixed with 20 ng of TFIH or 50 ng of Cak in 50 mM Tris (pH 7.6), 10 mM KCl, 10 mM MgCl₂, 2 mM dithiothreitol. After a 5-min preincubation at 30 °C, the substrate mix was added (100 ng of RNAPII and ATP to a final concentration of 10 μM, which included 5 μCi of [γ-32P]ATP). Kinase reactions were then performed for 15 min at 30 °C. Reactions were stopped by adding 25 μl of 2× gel-loading buffer (100 mM Tris (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol), and proteins were denatured at 100 °C for 5 min. Phosphorylated products were resolved in polyacrylamide gels and visualized in a PhosphorImager system.

Kinetics of Inhibition for cdk7 Phosphorylation of RNAPII—After a 5-min preincubation of TFIH with the indicated amounts of PC4, substrate mixes containing saturating amounts of ATP (including 10 μCi of [γ-32P]ATP) and varying amounts of RNAPII (from 25 to 250 ng as indicated) were added. Kinase reactions were performed as described above. The amounts of phosphorylation were measured in a PhosphorImager system using Molecular Dynamics software. A double-reciprocal plot is presented. To study inhibition of cdk-1 and cdk-2 by PC4, the phosphorylation reactions were similar to that for TFIH. Baculovirus-expressed cyclin A/cdk-1 or cyclin A/cdk-2 (27) were used as enzymes. Kinase reactions were performed for 10 min at 30 °C. The phosphorylation of substrates other than RNAPII was analyzed as described above, except that 100 ng of TFIIF (26), p53 (28), GST-cdk2 (2), or the indicated amount of Histone H1 (Sigma) were used as substrates. Kinase reactions were performed for 20 min at 30 °C. No PC4 or 37.5 or 75 ng of bacterially expressed PC4 was added as indicated.

PC4 Mutants—Mutant forms of PC4 were generated by inserting double-stranded DNA oligonucleotides, which contained the mutated codons, into baculovirus DNA, in appropriate restriction sites. Mutant forms of PC4 were then expressed and purified as described for the wild-type protein. Inhibition of cdk7 by mutant forms of PC4 was analyzed as described above, except that 90 ng of each of the different mutant or wild-type forms of PC4 were used. To analyze phosphorylation of wild-type or mutant forms of PC4, kinase reactions were performed as described above, except that RNAPII was omitted in the reactions. In these reactions, 90 ng of wild-type or mutant forms of PC4 were added as a component of the substrate mix together with ATP. Thus, PC4 or its mutant forms were not preincubated with TFIH before the addition of ATP.

Purification of Native PC4—240 μg of HeLa S100 extract (a side fraction of nuclear extract) was first fractionated on a phoshocellulose (P11) column. The column was then washed with BC500 (20 mM Tris-Cl (pH 7.9), 20% glycerol, 0.2 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and 500 mM KCl), and PC4-containing fractions were eluted with BC500 (containing 850 mM KCl). The eluate was then mixed with an equal volume of BC100 and fractionated on a
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To analyze the inhibition of RNAPII phosphorylation by PC4, recombinant PC4 was expressed in Echerichia coli and purified to homogeneity (Fig. 1A). The inhibitory activity of PC4 was analyzed using either TFIIH or the trimeric cdk7 kinase complex. Recombinant PC4 inhibited phosphorylation of the largest subunit of RNAPII by either TFIIH or the trimeric cdk7 complex (Fig. 1B). It is difficult to assess the change in the mobility of the largest subunit of RNAPII under our assay conditions. Interestingly, the concentration of PC4 required for inhibition of cdk7 kinase activity was similar to the concentrations of bacterially expressed P15 and P16 proteins previously reported to inhibit cdk4 and cdk6 (9). To analyze the mechanism of inhibition of RNAPII phosphorylation by PC4, kinetic assays were performed in which varying amounts of RNAPII were phosphorylated by a constant amount of TFIIH in the presence of excess ATP at several different concentrations of PC4. This analysis revealed that PC4 inhibited the phosphorylation of RNAPII in a competitive manner (Fig. 1C). The $K_I$ for PC4 inhibition of RNAPII phosphorylation was calculated to be 100 nM.

PC4 contains a ssDNA-binding domain, which maps to the C-terminal half of the protein (Fig. 2A). Additionally, the N-terminal half of PC4 encompasses two serine-rich domains, which contains the conserved phosphorylation sites in PC4, and a lysine-rich domain (Fig. 2A). To further analyze the inhibitory activity of PC4, we constructed a series of PC4 mutants (Fig. 2B). Mutant proteins were expressed in E. coli and purified to homogeneity (Fig. 2C). Analysis of the PC4 mutants for their inhibitory activity of RNAPII phosphorylation by cdk7 revealed that double (mutants 1 and 2) or triple (mutant 3) point mutations in the lysine-rich motif (amino acids 29–41) abolished the inhibitory activity of PC4 (Fig. 2D). In contrast, a point mutation in the C-terminal half of PC4 (mutant 5, Fig. 2D), or a quadruple point mutation in the major casine kinase II (CKII) phosphorylation site located in the first serine-rich domain (mutant 6, Fig. 2D), did not effect the inhibitory activity of PC4. Moreover, a triple point mutant C-terminal to amino acid 41 (Fig. 2D, mutant 4) retained the kinase inhibitory activity, although to a smaller extent than that of wild-type PC4 (Fig. 2D, compare WT with mutant 4). To rule out the possibility that inhibition of RNAPII phosphorylation was the result of PC4 acting as a competitive substrate of TFIIH, we analyzed the phosphorylation of PC4 and its mutants by TFIIH in the absence of RNAPII. As Fig. 2E indicates, all forms of PC4 were phosphorylated by TFIIH, although only wild-type and mutants 4, 5, and 6 inhibited RNAPII phosphorylation (Fig. 2D). Therefore, phosphorylation of PC4 by TFIIH does not correlate with its inhibitory activity toward RNA-PII phosphorylation.

It was previously reported that PC4 was subject to phosphorylation and that the phosphorylated form of PC4 was devoid of transcriptional coactivation activity (29). To investigate the possible role of PC4 phosphorylation on the cdk7 kinase inhibitory activity, we isolated mammalian PC4 from HeLa cells using conventional chromatography technique. Analysis of the last chromatography step (heparin-5PW), using SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining, revealed that the phosphorylated form of PC4 (PC4-P, 16 kDa) eluted at 0.3 M KCl (Fig. 3A, lane 2), whereas the unphosphorylated form (PC4, 15 kDa) eluted at 0.5 M (lane 3). The 0.4 M fraction contained a mix of both phosphorylated and unphosphorylated forms of PC4 (lane 1). To confirm that the 15- and 16-kDa polypeptides are PC4, the respective bands were cut from a colloidal blue-stained SDS-polyacrylamide gel, cleaved...
and unphosphorylated forms of PC4 (0.4M eluate) displayed phosphorylated form of PC4 (0.3M eluate) was devoid of kinase inhibitory activity (Fig. 3B lane 2).

The ssDNA binding domain (black boxes), and the lysine-rich domain (cdk inhibitory domain (CID), vertically striped box). The numbers above the boxes correspond to the PC4 amino acids as shown in panel B. B, single or multiple point mutants of PC4 (Mut.1-Mut.6) are shown. C, equal amounts (1 μg) of purified wild-type (WT) or each of the mutant forms of PC4 (mutants 1–6) were resolved in SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. The numbers at the top of the gel correspond to the PC4 mutants indicated in panel B. D, TFIIH-mediated phosphorylation of RNAPII was performed in the presence of no PC4 (--), 75 ng of bacterially expressed wild-type, or 80 ng of mutant forms (mutants 1–6) of PC4, as shown at the top of the panel. Phosphorylated products were resolved in SDS-polyacrylamide gels, dried, and visualized using a PhosphorImager system. E, phosphorylation of PC4 by TFIIH. Note that RNAPII was omitted from the reactions. The phosphorylated wild-type or mutant forms of PC4 (mutants 1–6) are shown.

We next tested whether PC4 inhibited the kinase activity of other cdks. Baculovirus-expressed cyclin A/cdk-1 and cyclin A/cdk-2 were purified to homogeneity and incubated with purified RNAPII in the absence or presence of 37.5 or 75 ng of purified recombinant PC4. Although PC4 efficiently inhibited phosphorylation of RNAPII by both kinases, we observed that phosphorylation of another protein present in the same reactions (tentatively identified as cyclin A) was not affected by the inhibitor (Fig. 4A). The inability of PC4 to inhibit the phosphorylation of cyclin A by cdk2 or cdk1 prompted us to analyze the effects of PC4 on the phosphorylation of other substrates of cdk1, cdk2, or cdk7. All naturally occurring cdk inhibitors are specific for one or more cdks but cannot discriminate among different substrates (9–16). In contrast, synthetic peptide cdk inhibitors (aptamers) have been described that selectively inhibit phosphorylation of only some cdk2 substrates (30). To determine whether PC4 is indeed a naturally occurring substrate-specific (“aptamer-like”) cdk inhibitor, the activity of PC4 on the phosphorylation of three other substrates of cdk1, cdk2, or cdk7 was examined. Although PC4 did not inhibit cdk7-dependent phosphorylation of the large subunit of TFIIH or p53, it inhibited phosphorylation of GST-cdk2 (Fig. 4B). To further test the specificity of PC4 kinase inhibitory activity, Histone H1 was used as a substrate for cyclin A/cdk-1, cyclin A/cdk-2, and cyclin B/cdk-1 in the presence of 0, 37.5, or 75 ng of purified PC4. Phosphorylation of either 1.0 μg (standard conditions) or 100 ng (same amount as RNAPII) of histone H1 by any of the three tested kinases was not inhibited by PC4 (Fig. 4C and data not shown), although similar concentrations of PC4 inhibited RNAII phosphorylation by cdk2 and cdk1 (Fig. 4A). These results demonstrate that PC4 behaved similarly to the previously described peptide aptamers and therefore represents a novel class of naturally occurring cdk inhibitors.

We show that PC4 acts as a substrate-specific inhibitor of
PC4 Inhibits RNAPII Phosphorylation

![Diagram of PC4 Inhibits RNAPII Phosphorylation](image)

Fig. 4. PC4 is a substrate-specific cdk inhibitor. A, kinase reactions were performed in the presence of 0 ( ), 37.5, or 75 ng of bacterially expressed PC4, using as kinase recombinant cyclin A/cdk-1 (lanes 1–3) or cyclin A/cdk-2 (lanes 4–6) expressed in insect cells. Phosphorylated products were resolved in polyacrylamide gels, dried, and visualized in a PhosphorImager system. The bands corresponding to RNAPII phosphorylated products were resolved in polyacrylamide gels, dried, and exposed. B and C, phosphorylation of RNAPII, p53, or GST-cdk2 by TFIIH in the presence of different amounts of PC4. The bands corresponding to the specific phosphorylation of TFIIF, p53, or GST-cdk2 by TFIIH in the presence of different amounts of PC4. The bands corresponding to RNAPII phosphorylated products were resolved in polyacrylamide gels, dried, and visualized. The bands corresponding to RNAPII phosphorylated products were resolved in polyacrylamide gels, dried, and exposed. C, phosphorylation of 1 μg or 100 ng of histone H1 by cyclin A/cdk-1 (lanes 1–3) or cyclin A/cdk-2 (lanes 4–6) in the presence of 0 ( ), 37.5, or 75 ng of PC4 was analyzed. Phosphorylated products were resolved in polyacrylamide gels, dried, and exposed.

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H. Ge, unpublished observations.
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J. Biol. Chem. 2000, 275:6071-6074.
doi: 10.1074/jbc.275.9.6071

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