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Phosphorylation negatively regulates the function of coactivator PC4

HUI GE*, YINGMING ZHAO†, BRIAN T. CHAIT†, AND ROBERT G. ROEDER*‡

*Laboratory of Biochemistry and Molecular Biology and †Laboratory for Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, New York, NY 10021

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ABSTRACT **Human positive cofactor 4 (PC4) mediates activator-dependent transcription by RNA polymerase II, apparently through interactions with transcriptional activators and the basal transcription machinery. We report here that PC4 function is modulated by *in vivo* phosphorylation. Protein–protein interaction studies and *in vitro* transcription assays demonstrate that only the nonphosphorylated form of PC4 is functionally active. Although recombinant PC4 can be phosphorylated by casein kinase II and protein kinase C *in vitro*, mutational and mass spectrometric analyses suggest that the *in vivo* hyperphosphorylation of PC4 is mediated mainly by casein kinase II and restricted to an N-terminal serine-rich region. These observations provide one example of a transcriptional cofactor that is negatively regulated by casein kinase II phosphorylation.**

In eukaryotic cells the transcription of genes encoding proteins is effected by RNA polymerase II in conjunction with general factors (TFIIA, -B, -D, -E, -F, -G/J, -H, and -I) that act through core promoter (TATA and initiator) elements (1, 2). In the common case, this process begins with recognition of the TATA element by the TATA binding protein (TBP) of TFIID and continues with the recruitment and function of the other factors (reviewed in refs. 1 and 2). Transcription initiation and elongation events are further regulated by gene-specific activators that function through distal control elements and, in many cases, consist of discrete DNA binding and activation domains (reviewed in ref. 3). However, in higher eukaryotes the function of transcriptional activators requires factors (coactivators) other than those that suffice for basal transcription, including TBP-associated factors (TAFs) within TFIID (reviewed in refs. 4 and 5) and positive cofactors (PCs) derived from the USA (upstream stimulatory activity) cofactor fraction (reviewed in refs. 6 and 7). In some cases, other gene- or activator-specific coactivators may also be required (see, for example, refs. 8–10). Mechanistic studies have shown physical and functional interactions of activators with general factors and coactivators as well as interactions between coactivators and general factors (refs. 6, 7, 11, 12, and references therein).

The functions of various transcription factors have also been shown to be subject to regulation by phosphorylation (reviewed in refs. 13 and 14), one of the most common post-translational modifications. In the case of the general transcription machinery, phosphorylation of the C-terminal domain of the largest subunit of RNA polymerase II has been correlated with the transition from initiation to elongation and the unphosphorylated form of RNA polymerase II has been shown to be preferentially recruited into preinitiation complexes, suggesting that phosphorylation of the C-terminal domain may regulate both of these events (reviewed in refs. 15 and 16). Examples of regulatory modifications of classical DNA binding activators include (*i*) mitosis-specific phosphorylation of the homeodomain of Oct-1, which correlates with inhibition of DNA binding to target genes such as histone H2B (17); (*ii*) protein kinase A-mediated phosphorylation of CREB, which is required for activation and physical interactions with the coactivator CBP (ref. 9 and references therein); and (*iii*) c-Jun NH_2-terminal kinase-mediated phosphorylation of c-Jun, which is considered to potentiate its trans-activation function (18).

We (6) and others (7) recently have described the purification and characterization of the USA-derived coactivator PC4. This factor mediates activation, in conjunction with TAFs, of a number of apparently distinct types of activation domains (6) and appears to act as an adaptor by virtue of its ability to interact with DNA-bound activation domains (6) and with a component (TFIIA) of the general transcriptional machinery (6, 7). Here we show that PC4 is subject to *in vivo* phosphorylation events that negatively regulate its *in vitro* coactivator function and its ability to interact with the activator VP16. Mutation and mass spectrometric analyses suggest that phosphorylation of PC4 *in vivo* is mediated mainly by casein kinase II (CKII), although several other protein kinases can also phosphorylate PC4 *in vitro*. Peptide mapping suggests that phosphorylation by CKII *in vivo* is restricted to seven serine residues at the N-terminal region. Possible mechanisms for the regulation of PC4 function by CKII phosphorylation are discussed.

MATERIALS AND METHODS

Protein–Protein Interactions. HeLa nuclear extract (19) (100 μl) was adjusted to an ionic strength equivalent to 200 mM KCl and incubated (4°C, 30 min) with 100 μg of either GST, GST-VP16, GST-VP16Δ456-FP442, or GST-VP16Δ456 protein immobilized on glutathione-Sepharose and preequilibrated with buffer D (19) containing 200 mM KCl. After washing with the equilibration buffer, the matrix was dissolved in SDS sample buffer, and bound proteins were resolved by 15% SDS/PAGE, blotted onto membranes, and probed with anti-PC4 polyclonal antibody.

Dephosphorylation and Phosphorylation Assays. Dephosphorylation assays were carried out in 20-μl reaction mixtures containing 2.5 μl of USA fraction (heparin-Sepharose fraction, 0.5 mg/ml) (5), 1× CIP (calf intestinal alkaline phosphatase) buffer (50 mM Tris·HCl, pH 8.7/1 mM $MgCl_2$/1 mM spermidine/0.1 mM $ZnCl_2$) and 1 or 2 units of CIP (BMB) or 2 units of heat-treated (100°C, 3 min) CIP at 37°C for 45 min. Reactions were stopped by adding an equal volume of 2× SDS sample buffer. After resolution by 15% SDS/PAGE

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Abbreviations: PC, positive cofactor; PC4-P, phosphorylated PC4; CKII, casein kinase II; CIP, calf intestinal alkaline phosphatase; GST, glutathione *S*-transferase; USA, upstream stimulatory activity; PKC, protein kinase C; HMK, heart muscle kinase; ssDNA, single-stranded DNA.

‡To whom reprint requests should be addressed.

and transfer to nitrocellulose, PC4 was detected by conventional immunoblot procedures with PC4 antibodies.

In vitro phosphorylation assays were performed at 30°C for 30 min in a 20-μl reaction mixture containing 200 μg of each individual protein, 100 μM ATP, and 10 μCi of [γ-^{32}P]ATP (1 Ci = 37 GBq). For the heart muscle kinase (HMK) assay, the reaction mixture also contained 20 mM Tris·HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol (DTT), 12 mM $MgCl_2$, and 1–5 units of HMK (Sigma). For the CKII assay, the reaction mixture contained 25 mM β-glycerophosphate (disodium salt, pH 7.5), 1 mM DTT, 8 mM $MgCl_2$, 0.5 mM K_2EDTA, 0.5 mM K_2EGTA, and 0.3 milliunit of recombinant CKII (Calbiochem). For the protein kinase C (PKC) assay, the reaction mixture contained 20 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM $CaCl_2$, and 0.15 milliunit of rat brain PKC (BMB). In some cases phosphorylated proteins were analyzed directly either by 15% SDS/PAGE followed by autoradiography (Fig. 3*B*) or by 18% SDS/PAGE followed by autoradiography (Fig. 4 *Upper*) or Coomassie blue staining (Fig. 4 *Lower*). In other cases phosphorylated proteins were bound to immobilized GST-VP16 before resolution by SDS–PAGE followed by autoradiography (Fig. 3*B*, lanes 5–8). Truncation mutant PC4(48–127) was constructed by inserting a 243-bp C-terminal fragment (filled *Taq* I–*Eco*RI) of PC4 into a pGEX-2T vector. PC4(1–47) was constructed by inserting a 141-bp N-terminal fragment (filled *Eco*RI–*Taq* I) into a pGEX-2T vector. Preparation of truncated recombinant proteins was essentially as described for the full-length protein (6).

Purification of Naturally Phosphorylated PC4. Naturally phosphorylated PC4 was purified from HeLa nuclear extracts through the heparin-Sepharose step essentially as described for the USA fraction (5, 6), except that the majority of phosphorylated PC4 was recovered from the 0.3 M KCl fraction instead of the 0.5 M KCl fraction. The 0.3 M fraction from heparin was further chromatographed on a single-stranded DNA (ssDNA) agarose column. After washing with BC500 (500 mM KCl), phosphorylated PC4 (PC4-P) was eluted with BC1000 and dialyzed against BC100. PC4 samples were incubated without or with 1 unit (per 10 μg of PC4) of CIP or heat-inactivated CIP and analyzed, along with recombinant PC4 purified from bacteria, by SDS/PAGE and Coomassie blue staining.

Mass Spectrometric Analyses. Matrix-assisted laser desorption mass spectrometric analysis was carried out on a laser desorption time-of-flight instrument constructed at the Rockefeller University (20). Positive ion mass spectra were collected by adding individual spectra obtained from 200 laser shots. Phosphorylated and dephosphorylated PC4 were prepared for laser desorption mass analysis as follows. The laser desorption matrix material [25–50 mM 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid)] was dissolved in 0.1% aqueous trifloroacetic acid/acetronitrile, 2:1 (vol/vol). The natural PC4 samples were mixed with the matrix solution to give a protein concentration of ≈4 μM. A small aliquot of the sample matrix solution (containing 2 pmol of the protein) was applied to the mass spectrometer sample probe tip, dried at 25°C, and inserted into the spectrometer for analysis. Bovine cytochrome *c* was used to calibrate the mass spectra. Electrospray ionization mass spectrometric analysis was carried out on a Finnigan-MAT TSQ 700 mass spectrometer fitted with a Finnigan electrospray ion source. Proteins (5–10 μM) were electrosprayed from a solution of 1% aqueous acetic acid/methanol, 1:1 (vol/vol), at a rate of 3–4 μl/min.

RESULTS

Phosphorylation of PC4 Inhibits Its Activity To Interact with VP16. We have recently reported the isolation and characterization of a human positive cofactor, PC4, that markedly stimulates activator-dependent transcription without a significant effect on basal (core promoter) transcription. Immunoblot analyses have shown that most mammalian cells contain two electrophoretically distinct forms of PC4. In HeLa nuclear extracts the slower migrating form is about 10- to 20-fold more abundant than the faster migrating form (Fig. 1*A*, lane 5). To test whether both forms of natural PC4 are able to interact with the activation domain of VP16, as previously shown for recombinant PC4 (6), crude nuclear extracts were applied to columns containing immobilized GST-VP16 fusion proteins and the bound proteins were resolved electrophoretically and monitored by anti-PC4 antibodies. As shown in Fig. 1*A*, only the high mobility form of PC4 was retained by the fusion protein with an intact VP16 activation domain (lane 2), whereas neither form of PC4 was stably bound to fusion proteins with completely inactive (lane 3) or partially active (lane 4) VP16 activation domains or to GST alone (lane 1). In further analyses with a partially purified cofactor fraction (USA) (5, 6, 21) containing both forms of PC4 (Fig. 1*B*), incubation with CIP (lanes 3 and 4), but not with heat-inactivated CIP (CIPΔ, lane 2) or buffer (lane 1), quantitatively converted the lower mobility form of PC4 to the high mobility form. Thus, the low mobility form appears to be a phosphorylated species of PC4 that is unable to interact with the VP16 activation domain *in vitro*.

Phosphorylation of PC4 Negatively Regulates Its Coactivator Function. These results, plus the observation that recombinant PC4 (produced in *Escherichia coli*) is largely unmodified but fully active in potentiation of activated transcription *in vitro* (6), suggested that the naturally phosphorylated form of PC4 might be transcriptionally inactive. To test this assumption, PC4-P from HeLa cells was purified to homogeneity (Fig. 2*A*, lane 2), treated with either active CIP or heat-inactivated CIP, further repurified on ssDNA columns, and analyzed, along with recombinant PC4, by SDS/PAGE (Fig. 2*A*) and in transcription assays (Fig. 2*B*). Recombinant PC4 and PC4 dephosphorylated by CIP potentiated GAL4-AH-

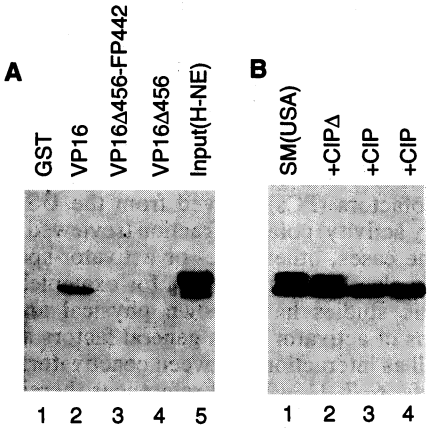

FIG. 1. PC4 interaction with VP16 is regulated by phosphorylation. (*A*) Interactions of different forms of natural PC4 with VP16 activation domains. PC4 in HeLa nuclear extract (Input, lane 5) was tested for binding either to immobilized glutathione *S*-transferase (GST) (lane 1) or to immobilized GST fused to an intact (residues 413–490) and fully active VP16 activation domain (lane 2), to a truncated activation domain with a point mutation (VP16Δ456-FP442) that is completely inactive (lane 3), or to a truncated activation domain (VP16Δ456) that is partially active (lane 4). Bound proteins were detected with antiserum against recombinant PC4. For details regarding activation domain mutants see ref. 6 and references therein. (*B*) Effects of dephosphorylation of PC4. Variant forms of PC4 in a partially purified PC4-containing USA fraction (lane 1, starting material) were analyzed by immunoblot following incubation with 2 units of heat-activated CIP (CIPΔ) (lane 2) or with 1 unit (lane 3) or 2 units (lane 4) of active CIP.

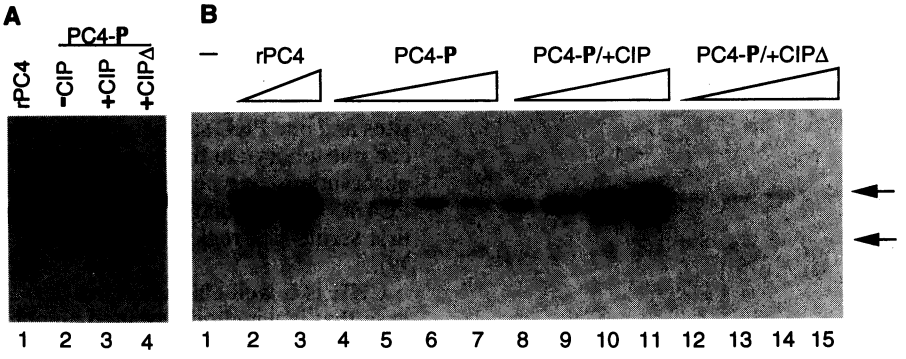

FIG. 2. Potentiation of transcriptional activation by PC4 is inhibited by phosphorylation. (*A*) Purified recombinant PC4 (lane 1) and naturally phosphorylated PC4 (PC4-P) treated with buffer alone (lane 2), with active CIP (lane 3), or with heat-inactivated CIP (lane 4) were analyzed by SDS/PAGE and Coomassie blue staining. (*B*) Functional analysis of naturally phosphorylated and dephosphorylated PC4. *In vitro* transcription assays were carried out with GAL4-AH and increasing amounts of either recombinant PC4 (lanes 2 and 3) or naturally phosphorylated PC4 incubated with buffer alone (lanes 4–7), with active CIP (lanes 8–11), or with heat-inactivated CIP (lanes 12–15). Upper and lower arrows indicate RNA products derived from activator-responsive (pG_5HMC_2AT) and core promoter (pMLΔ53) templates, respectively. *In vitro* transcription assays with general transcription factors, GAL4-AH, PC4, and control and activator-responsive templates were performed as described (6). The amounts of PC4 presented in the reactions were approximately 20 ng (lanes 4, 8, and 12), 40 ng (lanes 5, 9, and 13), 80 ng (lanes 2, 6, 10, and 14), and 160 ng (lanes 3, 7, 11, and 15).

activated transcription, while no such activity was detected with either PC4-P or PC4-P treated with inactive CIP (Fig. 2*B*). These data clearly show that phosphorylation of PC4 negatively regulates its activity.

CKII and PKC Inhibit PC4 Binding to VP16. A search of the PC4 sequence for protein kinase consensus sites (22, 23) revealed seven potential CKII sites within the first serine-rich region, an additional six CKII sites distributed in the rest of the molecule, and four potential PKC sites (Fig. 3*A*). A recombinant PC4 (PC4-K) containing an added HMK site at the N terminus of PC4 (6) could be labeled *in vitro* by HMK, CKII, and PKC, although the efficiency of PKC phosphorylation was relatively low (Fig. 3*B*). Only PC4 phosphorylated by HMK was able to bind to GST-VP16, whereas phosphorylation by CKII, PKC, or CKII and PKC together abolished the ability of PC4 to bind to GST-VP16 (Fig. 3*B*). These observations suggest that the natural (*in vivo*) phosphorylation of PC4 may be brought about by CKII or PKC or both.

PC4 Activity Is Mediated by CKII *in Vivo*. A mutational analysis showed that CKII can phosphorylate a fragment of PC4 containing the N-terminal 47 amino acid residues [PC4(1–47)], but not a fragment containing the C-terminal 80 residues [PC4(48–127)], whereas the reverse is true for PKC (Fig. 4 *Upper*). An analysis of the kinase-treated proteins by Coomassie blue staining (Fig. 4 *Lower*) indicated that recombinant PC4 phosphorylated by CKII resulted in a significant mobility shift comparable to that observed *in vivo*, whereas PC4 phosphorylated by PKC did not significantly alter the mobility. Somewhat surprisingly, CKII phosphorylation of PC4 (1–47) did not alter the mobility of the polypeptide, possibly reflecting neutralization by phosphate groups of the highly charged N-terminal region (21% serine and 28% lysine plus arginine). These observations indicate that PC4 is phosphorylated mainly by CKII *in vivo*.

Matrix-assisted laser desorption mass spectral analysis (24) of phosphorylated and dephosphorylated PC4 further reinforced the above observations (Fig. 5). The measured molecular mass of dephosphorylated PC4 (14,266 ± 4 Da) corresponded closely with the mass calculated from the cDNA-derived sequence of PC4 with deletion of the N-terminal methionine residue (14,263 Da). The absence of modifications in dephosphorylated PC4 was also confirmed by matrix-assisted laser desorption mass spectrometric peptide mapping after digestion with Endo-Arg-C protease and CNBr cleavage (data not shown). The phosphorylated form of the protein was determined to have a molecular mass (14,818 ± 4 Da) that was 552 ± 4 Da higher than dephosphorylated PC4, consistent with the presence of an average of seven phosphate moieties (80 Da per moiety). A more detailed investigation of PC4-P was carried out using electrospray ionization mass spectrometry (24) in which the individual phosphorylation states were well resolved. The majority of the protein

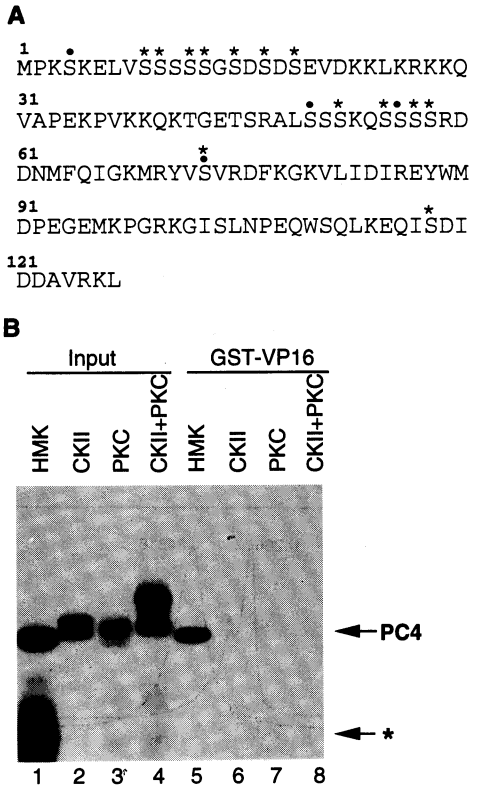

FIG. 3. Phosphorylation of PC4 by CKII and PKC. (*A*) Predicted protein kinase sites present in PC4 sequence. Dots indicate potential PKC sites; asterisks indicate potential CKII sites. (*B*) *In vitro* phosphorylation of PC4 by either CKII or PKC inhibits PC4 binding to VP16. Lanes 1–4, direct analyses (SDS/PAGE plus autoradiography) of PC4-K phosphorylated by HMK, CKII, PKC, or CKII and PKC as indicated. Lanes 5–8, phosphorylated PC4 retained by immobilized GST-VP16. An asterisk indicates an unidentified product of the HMK reaction (possibly a contaminating HMK substrate in the enzyme).

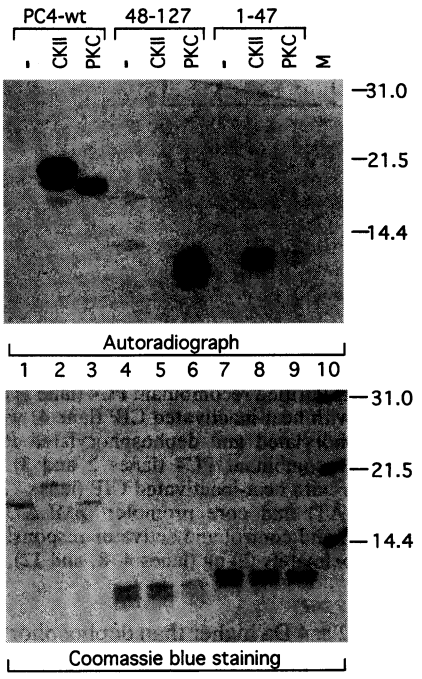

FIG. 4. *In vitro* phosphorylation of PC4 and derived fragments. (*Upper*) Autoradiograph of PC4-P and truncation mutants resolved by SDS/PAGE. (*Lower*) Same gel visualized by Coomassie blue staining. Full-length recombinant PC4, PC4(48–127) (corresponding to an N-terminal truncation), and PC4(1–47) (corresponding to a C-terminal truncation) were incubated without any protein kinase (lanes 1, 4, and 7), with CKII (lanes 2, 5, and 8), or with PKC (lanes 3, 6, and 9), respectively. M, protein molecular mass standards in kDa.

was found to contain seven phosphorylated amino acid residues, although small proportions were identified with six and eight phosphates. Matrix-assisted laser desorption mass spectrometric peptide mapping of PC4-P (using CNBr cleavage and Endo-Lys-C protease digestion) indicated no significant phosphorylation between residues 29 and 127 (data not shown). We deduce that the majority of the seven phosphate residues present in PC4 are located in residues 2–28.

DISCUSSION

In summary, we have observed (*i*) that the *in vivo* phosphorylation of PC4 negatively regulates its coactivator activity and its ability to interact with the activator VP16, (*ii*) that the *in vivo* phosphorylation of PC4 involves modification of seven N-terminal residues between positions 2 and 28, and (*iii*) that, although sequence analysis predicts seven CKII sites and one PKC site between residues 2 and 28, only CKII can phosphorylate the N-terminal fragment. Based on these observations, we conclude that the coactivator function of PC4 *in vivo* is regulated by CKII hyperphosphorylation of the first serine-rich region (at residues 9, 10, 12, 13, 15, 17, and 19).

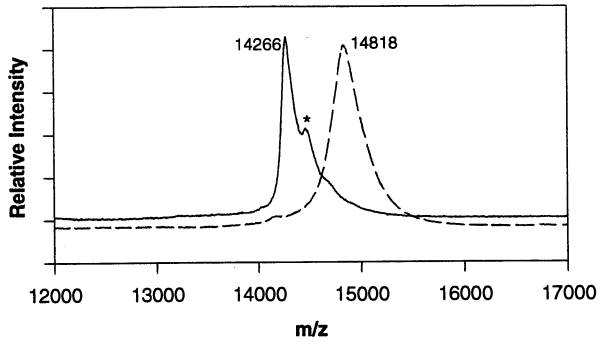

FIG. 5. Positive ion matrix-assisted laser desorption ionization mass spectra of dephosphorylated PC4 (solid line) and PC4-P (dashed line). The peak labeled "*" arises through adduction of the sinapinic acid matrix to unphosphorylated PC4 (25). No matrix adduction peak was identified in the mass spectrum of PC4-P owing to phosphorylation heterogeneity of the protein (see text). The molecular masses of dephosphorylated PC4 and PC4-P were determined to be 14,266 ± 4 Da and 14,818 ± 4 Da, respectively.

CKII is a well-characterized Ser/Thr-specific protein kinase that is widely distributed among eukaryotic organisms (26). Phosphorylation by CKII can either negatively or positively regulate the DNA binding activity of transcription factors such as c-Myb (27), Max (28), and SRF (29). Phosphorylation by CKII of the C-terminal region of UBF has also been suggested to play an important role in growth-dependent control of rRNA synthesis by RNA polymerase I (30). However, in the case of PC4, phosphorylation by CKII abolishes its interaction with an activation domain as well as its ability to mediate activator-dependent transcription. Since we have not observed a significant change in the general DNA binding ability of PC4 by phosphorylation (H.G. and R.G.R., unpublished data), it seems likely that phosphorylation alters the conformation of PC4 and, in turn, its ability to interact with VP16. Results reported by Kretzschmar *et al.* (7) after completion of this work also showed inhibitory effects of phosphorylation on coactivator function and on the ability of PC4 to interact with the basal transcriptional machinery. Further mutational and crystallographic analyses of phosphorylated and unphosphorylated forms of PC4 may provide additional information regarding the mediation of transcriptional regulation by phosphorylation.

As we previously reported (6), other ssDNA binding proteins cannot replace the PC4 coactivator activity. The phosphorylated form of PC4 retains DNA binding activity but lacks the ability to interact with activation domains or general factors and to induce activated transcription, further demonstrating that the ssDNA binding activity of PC4 is not sufficient for its coactivator function. Yeast ADR1, which activates ADH2 expression, provides another example of a transcription factor whose function is inhibited by phosphorylation (31, 32). As described for PC4, however, this phosphorylation appears to inhibit a secondary function, rather than the (site-specific) DNA binding ability. In the case of PC4, the secondary function could serve mainly to recruit PC4 to the promoter, while the ssDNA binding activity might function in some critical step in promoter activation (e.g., in stabilizing a single-stranded region of DNA during open complex formation; see ref. 33 and references therein). Although global changes in the level of PC4 phosphorylation might simply reflect a more global regulatory mechanism, it is also possible that PC4 undergoes phosphorylation while in the initiation or early elongation complex; such a periodic change in phosphorylation state during the transcription cycle would resemble that proposed for the C-terminal domain of RNA polymerase II.

Our results indicate that ≈95% of the total cellular PC4 is phosphorylated by CKII and thus inactive *in vivo*, while only 5% is in the nonphosphorylated active form. Therefore, the regulation of CKII activity may represent a crucial step in the regulation of PC4 function. Since it has been suggested that the activity of CKII can be rapidly enhanced by insulin and epidermal growth factor (34), it will be of interest to investigate whether PC4-mediated transcription is activated in response to such signals in various signal transduction pathways.

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