Phosphorylation Modulates the Function of the Vasoactive Intestinal Polypeptide Receptor Transcriptional Repressor Protein*

(Received for publication, August 27, 1999, and in revised form, September 30, 1999)

Lin Pei‡
From the Division of Endocrinology and Metabolism, Cedars-Sinai Research Institute-UCLA School of Medicine, Los Angeles, California 90048

The transcriptional repressor for rat vasoactive intestinal polypeptide receptor 1 (VIPR-RP) is a recently isolated transcription factor. In this study, we have characterized the functional domains of VIPR-RP and the importance of phosphorylation on VIPR-RP function. Using various regions of VIPR-RP in gel mobility shift assays, we show that the amino acid sequences between positions 367 and 475 play an essential role for VIPR-RP DNA binding. Transient transfection of fusion constructs containing GAL4 DNA binding domain and different parts of VIPR-RP indicated that there are two separate transcriptional repression domains in VIPR-RP, located between amino acids 50 and 101 and between 469 and 527. We demonstrated that VIPR-RP is phosphorylated in vitro by casein kinase II on Ser-69/71 and Thr-110, and by cAMP-dependent kinase on Ser-245/361. Furthermore, by site-directed mutagenesis, we show that phosphorylation of the casein kinase II sites potentiates VIPR-RP transcriptional repression activity by enhancing its nuclear translocation, and that phosphorylation by cAMP-dependent kinase inhibits VIPR-RP transcriptional repression function without affecting its subcellular localization. These observations suggest that phosphorylation plays an important role in regulating VIPR-RP function.

The rat type 1 vasoactive intestinal polypeptide receptor (VIPR 1)* is expressed in multiple tissues throughout rat embryonic development and in the adult rat (1–3). The expression of VIPR 1 gene is regulated by interactions of multiple transcription factors with its 5′-regulatory sequences (4–6). While glucocorticoid receptor and Sp1 protein are required for VIPR 1 gene basal transcription activation (4, 5), VIPR repressor protein (VIPR-RP), a recently characterized transcription factor, represses VIPR 1 basal transcription (6).

VIPR-RP belongs to a family of proteins that includes differentiation-specific element-binding protein (DSEB) (7), the large subunit of murine activator 1 complex (A1p145) (8), mouse replication factor C140 (mRFC140) (9), PO-GA (10), and PCRH-REB (11). A common feature of these proteins is that they all contain a region of about 80 amino acids that shares high identity to bacterial ligases (12, 13). However, VIPR-RP encodes a much smaller protein than DSEB/A1p145, due to a single base deletion at amino acid 581 in VIPR-RP that results in a frameshift and a termination codon at amino acid 657 (6). DSEB was shown to bind to an enhancer element in angiotensinogen promoter that mediates the irreversible induction of transcriptional activation during differentiation of 3T3-L1 adipoblasts to adipocytes (7). A1p145/RFC forms part of a heteropentameric protein complex that is essential for DNA replication (14). These observations suggest that this family of proteins may play a dual role as transcription factor as well as a component of the DNA replication complex.

We showed in the previous study that VIPR-RP binds to VIPR 1 repressor element specifically and mediates transcriptional repression of the reporter plasmid containing four copies of its binding sequence (6). However, the functional domains responsible for VIPR-RP-specific DNA binding and transcriptional repression have yet to be defined. In this study, we have mapped the amino acid sequences required for VIPR-RP DNA binding by analyzing various regions of VIPR-RP either synthesized in vitro or expressed as GST fusion proteins, using gel mobility shift assays. Because many sequence-specific DNA-binding proteins have modular structure (15), we have determined the transcription repression domains of VIPR-RP by co-transfection of COS-7 cells with chimeras containing different parts of VIPR-RP fused to GAL4 DNA binding domain, together with a reporter gene containing GAL4 binding sites.

Protein phosphorylation is an important mechanism that modulates the activity of many transcription factors. Several putative phosphorylation sites for protein kinase A (PKA) and casein kinase II are present in VIPR-RP. In this study, the functional importance of phosphorylation of VIPR-RP by casein kinase II (CK-II) and PKA was investigated. We showed that VIPR-RP is phosphorylated by both kinases in vitro, and we have identified the Ser and Thr residues responsible for phosphorylation by mutagenesis studies. Finally, we demonstrated that phosphorylation by PKA inhibits the ability of VIPR-RP to repress transcription without affecting it subcellular localization, and that phosphorylation by CK-II enhances VIPR-RP transcriptional repression and nuclear localization.

MATERIALS AND METHODS

Plasmids—The reporter plasmid (USAJKLUC) containing two GAL4 binding sites inserted in front of thymidine kinase minimal promoter linked to luciferase (16) was kindly provide by Dr. V. K. Chatterjee (University of Cambridge, Cambridge, United Kingdom). The construction of expression vector containing GAL4 DNA binding domain (pGAL1–147) was described previously (17). The various pGAL–VIPR-RP expression vectors were generated by insertion of the corresponding DNA fragments from pCMV-VIP-RP coding for indicated sequence-specific DNA-binding proteins.

This paper is available on line at http://www.jbc.org

* This work was supported by National Institutes of Health Grant DK-02346 and American Lung Association Grant RG-018-N. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked " in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Div. of Endocrinology and Metabolism, Cedars-Sinai Medical Center, 8700 Beverly Blvd., D3066, Los Angeles, CA 90048. Tel.: 310-423-7682; Fax: 815-352-6253; E-mail: pei@cshs.org.
‡ The abbreviations used are: VIPR, vasoactive intestinal polypeptide receptor; VIPR-RP, transcriptional repressor for rat vasoactive intestinal polypeptide receptor 1; CK-II, casein kinase II; TK, thymidine kinase; PKA, cAMP-dependent protein kinase; NES, 4-morpholinobenzethanesulfonic acid; GFP, green fluorescent protein; NLS, nuclear localization signal; GST, glutathione S-transferase; DSEB, differentiation-specific element-binding protein; PCR, polymerase chain reaction; TBS, Tris-buffered saline; TBST, Tris-buffered saline with Tween 20.
amino acids into the polylinker of pGAL1–147. Deletion mutants were generated using ExSite PCR-based site-directed mutagenesis kit (Stratagene), and point mutations were made using QuikChange site-directed mutagenesis kit (Stratagene), following manufacturer’s instructions. The green fluorescence protein (GFP) and VIPR-RP fusion constructs were created by inserting a KpnI-EcoRV fragment from pCMV-VIPR-RP into KpnI-SmaI site of the GFP-C2 expression vector (CLONTECH). To construct glutathione S-transferase (GST) and VIPR-RP fusion protein, the coding region of VIPR-RP was amplified by PCR and cloned in frame at the EcoRI site of pGEX-4T-1 (Amersham Pharmacia Biotech) vector. Various deletions of GST-VIPR-RP were made using restriction enzyme digestion and ExSite PCR-based site-directed mutagenesis kit (Stratagene). The PKA C-subunit expression plasmid was kindly provided by Dr. Stanley McKnight (University of Washington, Seattle, WA).

Cell Culture and Transfections—COS-7 and F9 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% or 15% fetal bovine serum, respectively. Cells were transfected by calcium precipitation as described previously (5). Each DNA construct was transfected in triplicate and tested in at least three independent experiments. Transfection efficiency was monitored by co-transfecting thymidine kinase promoter linked to chloramphenicol acetyltransferase. Forty-eight hours after transfection, cell lysate was prepared and assayed for luciferase and chloramphenicol acetyltransferase activity as described previously (5).

In Vitro Transcription and Translation—Various regions of VIPR-RP were cloned into BlueScript vector (Stratagene) and were transcribed from T3 promoter and translated in reticulocyte lystate using TNT coupled reticulocyte lystate system (Promega). A typical reaction contains 25 μl of rabbit reticulocyte lysate, 2 μl of reaction buffer, 20 μM amino acid mixture, 1 μg of DNA template, 40 units of ribonuclease inhibitor, 10 units of T7 RNA polymerase, and 1 μl of Transcend™ biotin-lysyl-tRNA (Promega), in a total volume of 50 μl. The reactions were carried out at 30 °C for 1 h. Two microliters from each reaction were boiled in loading buffer and separated on 10% SDS-polyacrylamide gels. Gels were transferred to nylon membranes and blocked by incubation with Tris-buffered saline containing 0.5% Tween 20 (TBST). The membranes were incubated with streptavidin-horseradish peroxidase conjugate in TBST for 45 min, washed four times with TBST, and three times with TBS. The membranes were then incubated with the chemiluminescent substrate mixture for 1 min and exposed to Kodak x-ray film for 2 min.

Gel Mobility Shift Assay—A 42-base pair oligonucleotide containing the binding site for VIP-RP was end-labeled with ϕ-32P-ATP using T4 polynucleotide kinase. Binding reactions were performed in 20 μl of binding buffer (20 mM Tris, pH 7.5, 0.1 mM EDTA, 2 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol, 10% glycerol, and 1 μg of nonspecific competitor poly(dI-dC)) with either 2 μl of in vitro translated protein or 1 μg of recombinant VIPR-RP expressed in and purified from Escherichia coli. Binding was 15 min at room temperature. The anti-VIPR-RP antibody was added, and the binding was continued on ice for an additional 30 min. The reactions were electrophoresed on 4% nondenaturing polyacrylamide gel, dried, and exposed to x-ray film for 3–6 h.

In Vitro Kinase Assay—E. coli B21 cells carrying fusion protein of GST and various regions of VIPR-RP were grown in 2 ml of 2× YT medium to an A600 of 0.6–0.8. The expression of the fusion proteins was induced by 0.5 mM isopropyl-β-D-thiogalactoside for 2 h. Cells were collected by centrifugation and resuspended in 500 μl of ice-cold phosphate-buffered saline containing 100 μg/ml lysozyme. Cells were lysed by three freeze-thaw cycles. The cell lysate was centrifuged, and 20 μl of a 50% slurry of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) was added to the supernatant and incubated overnight at 4 °C. The Sepharose beads were sedimented. For CK-II assay, the beads were washed five times with CK-II buffer. The suspension was then incubated with 10 μCi of γ-32P-ATP (NEN Life Science Products, Boston, MA) and 0.1 milliunit of recombinant CK-II (Roche Molecular Biochemicals) at 37 °C for 1 h. The beads were then washed five times with the CK-II buffer and resuspended in SDS-loading buffer. The samples were boiled for 5 min, resolved on a 12% SDS-polyacrylamide gel, and subjected to autoradiography.

Fluorescence Microscopy—COS-7 cells transfected with GFP-VIPR-RP or GFP-VIPR-RP mutant expression vectors were fixed 24 h after transfection with 2% neutral buffered formaldehyde (2% formalde Vy, 20 mM NaPO4, pH 7.4) for 15 min at 37 °C and washed three times with phosphate-buffered saline. Slides were then examined with fluorescence microscope.

RESULTS

Identification of VIPR-RP DNA Binding Domain—Because VIPR-RP does not contain typical DNA binding motifs such as zinc fingers or basic leucine zipper, as present in many transcription factors, we sought to identify the region of VIPR-RP that is involved in its binding to DNA. Various regions of VIPR-RP were transcribed and translated in vitro (Fig. 1A), and the ability of the in vitro translated proteins to bind to VIPR-RP recognition sequence was tested in gel mobility shift assays. As shown in Fig. 2B, deletion of the C-terminal 46 amino acids did not affect VIPR-RP DNA binding (lane 1–610). However, when 266 amino acids were deleted from the C terminus, the truncated protein no longer bound to DNA (lane 1–390). Deletion up to 367 amino acids from the N terminus did not have effect on VIPR-RP DNA binding (lane 178–656 and 367–656). These results suggest that the region between amino acids 367 and 610 is sufficient to confer VIPR-RP DNA binding.

To further define the VIPR-RP DNA binding domain, the region between amino acids 367 and 475 was expressed in E. coli as a GST fusion protein. When the purified recombinant protein was used in gel mobility shift assay, it...
The DNA sequence encoding amino acids 367–475 and 476–527 were cloned into pGEX-4T prokaryotic expression vector. The GST-VIPR-RP construct with the deletion of an additional 200 amino acids from the C terminus (Fig. 3, construct 1–177) was able to repress the expression of the reporter gene. Similarly, deletion of the N-terminal 177 amino acids resulted in complete loss of transcriptional repression (Fig. 3, construct 178–656), but construct with further deletion (Fig. 3, construct 378–656) showed strong repression function. These results showed that inclusion of amino acids 178–377 in either N-terminal (construct 178–656) or C-terminal (construct 1–377) deletion mutant resulted in loss of transcriptional repression. However, deletion of this region, as in constructs 1–177 and 378–656, restored repression function. These results suggest that VIPR-RP contains two independent transcriptional repression domains located between amino acids 1 and 177 and between 378 and 656, and that the region between amino acids 178 and 377 suppresses VIPR-RP transcriptional repression function. The latter observation was confirmed by transfecting COS cells with fusion construct with the deletion of amino acids 178–378. Deletion of this region resulted in enhanced transcriptional repression activity of VIPR-RP (Fig. 3, construct Δ178–378).

To further define the amino acid sequences required for VIPR-RP transcriptional repression, detailed deletions were made within the regions between amino acids 1 and 177 and between 378 and 656. As shown in Fig. 3, deletion between amino acids 101 and 177 resulted in a slight increase in transcriptional repression (Fig. 3, construct 1–101), and further deletion of the N-terminal 49 amino acids had little effect on repression activity (Fig. 3, construct 50–101). Deletion of the amino acids between 378 and 527 resulted in complete loss of transcriptional repression (Fig. 3, construct 527–656). However, deletion of the C-terminal 129 amino acids did not affect repression activity (Fig. 3, construct 378–527). Within the region between amino acids 378 and 527, the C-terminal half (Fig. 3, construct 469–527) conferred transcriptional repression at the same level as construct 378–527, whereas the N-terminal half (Fig. 3, construct 378–469) showed much reduced repression activity. These results suggest that the transcriptional repression domains of VIPR-RP is located between amino acids 50 and 101 and between 469 and 527.

VIPR-RP Is Phosphorylated by CK-II in Vitro—The N-terminal transcriptional repression domain of VIPR-RP contains two clusters of acidic amino acids interspersed with Ser and Thr residues (amino acids D68SDSESEE75, and S108ETDEDDD115), which correspond to consensus phosphorylation sites for CK-II (20, 21). We sought to determine whether phosphorylation by CK-II has any effect on VIPR-RP function. Initially, we tested whether CK-II can phosphorylate VIPR-RP in vitro. The GST fusion protein containing either VIPR-RP amino acids 1–177 or 178–656 were expressed in E. coli, purified using glutathione-Sepharose, and incubated with recombinant CK-II in the presence of [γ-<sup>32</sup>P]ATP. As shown in Fig. 4A, the fusion protein containing VIPR-RP amino acids 1–177 was phosphorylated by CK-II, whereas the fusion protein containing amino acids 178–656 was not phosphorylated. To test which of the Ser and Thr residues are phosphorylated by CK-II, site-directed mutagenesis was used to change Ser-69, -71, and -108, as well as Thr-110, to alanines. As shown in Fig. 4B, substitution of Thr-110 resulted in about 40% reduction in VIPR-RP phosphorylation, whereas substitution of Ser-108 alone did not have any effect. When both Ser-69 and -71 were changed to alanine, VIPR-RP phosphorylation level was dramatically reduced. The mutant containing all three substitutions at Ser-69/71 and Thr-110 was no longer phosphorylated by CK-II. These results indicate that VIPR-RP is phosphorylated by CK-II on Ser-69/71 and Thr-110.

Phosphorylation of the CK-II Sites Enhances VIPR-RP Transcriptional Repression Domains—To localize the transcriptional repression domain of VIPR-RP, fusion constructs were made between various regions of VIPR-RP and the GAL4 DNA binding domain (GAL4-DBD, amino acids 1–147). The GAL4-DBD contains signals for dimerization (18) and nuclear translocation (19) in addition to its specific DNA binding activity, and shows no transactivation function. Therefore, VIPR-RP deletions can be analyzed for transcriptional regulation even when dimerization and nuclear translocation domains are deleted. The expression plasmids coding for GAL4-DBD fused to various parts of VIPR-RP were transfected into COS-7 cells together with a reporter plasmid containing two GAL4 binding sites in front of TK promoter-luciferase fusion (16). As shown in Fig. 3, deletion of the 279 amino acids from the C terminus (Fig. 3, construct 1–377) resulted in loss of transcriptional repression. However, generated a mobility retarded band (Fig. 2, lane 2), whereas no mobility shifted band was observed in the presence of GST (Fig. 2, lane 1). Furthermore, the mobility shifted band was competed by unlabeled homologous oligonucleotide (Fig. 2, lane 3), but not by oligonucleotide containing point mutations of the VIPR-RP binding site (Fig. 2, lane 4). Addition of anti-VIPR-RP antibody resulted in a supershifted band (Fig. 2, lane 5). Similar experiments were performed using GST fusion protein containing VIPR-RP amino acids 476–610, no DNA binding activity was observed for this fusion protein (Fig. 2, lane 6). These results suggest that the region between amino acids 367 and 475 is required for VIPR-RP DNA binding.

Identification and Characterization of the Transcriptional Repression Domains—To localize the transcriptional repression domain of VIPR-RP, fusion constructs were made between various regions of VIPR-RP and the GAL4 DNA binding domain (GAL4-DBD, amino acids 1–147). The GAL4-DBD contains signals for dimerization (18) and nuclear translocation (19) in addition to its specific DNA binding activity, and shows no transactivation function. Therefore, VIPR-RP deletions can be analyzed for transcriptional regulation even when dimerization and nuclear translocation domains are deleted. The expression plasmids coding for GAL4-DBD fused to various parts of VIPR-RP were transfected into COS-7 cells together with a reporter plasmid containing two GAL4 binding sites in front of TK promoter-luciferase fusion (16). As shown in Fig. 3, deletion of the 279 amino acids from the C terminus (Fig. 3, construct 1–377) resulted in loss of transcriptional repression. However,
Expression Plasmds

| Expression Vectors | Repression |
|--------------------|-----------|
| VIPR-Rep | 7.6 |
| GAL | 1.0 |
| GAL-Rep-1-377 | 1.03 |
| 1-177 | 8.4 |
| 1-101 | 10.5 |
| 50-101 | 10.1 |
| 178-656 | 0.99 |
| 378-656 | 10.7 |
| 527-656 | 1.2 |
| 378-527 | 10.2 |
| 378-469 | 3.8 |
| 469-527 | 9.6 |
| A178-378 | 15.2 |

**Phosphorylation and Transcription Repression**

**Protein Kinase A Phosphorylates VIPR-RP in Vitro**—Cyclic AMP (cAMP) mediates hormonal stimulation of a variety of eukaryotic genes (22). Since most of known cellular effects of cAMP occur via the catalytic subunit (C-subunit) of cAMP-dependent kinase (PKA), it is likely that this enzyme mediates the phosphorylation of factors that are critical for transcriptional response. The presence of multiple potential phosphorylation motifs (23) in VIPR-RP prompted us to ask whether this transcription factor is phosphorylated by PKA.

The GST fusion protein containing various parts of VIPR-RP were expressed in E. coli, purified using glutathione-Sepharose, and incubated with recombinant C-subunit of PKA, whereas fusion proteins containing amino acids 1–177 and 378–656 were not phosphorylated. These results suggest that the phosphorylation sites for PKA are located between amino acids 178–377. Within this region, there are five potential Ser and Thr residues that can be phosphorylated by PKA. To determine which of these residues is involved in PKA phosphorylation, site-directed mutagenesis was performed to change each one of these residues to alanine. As shown in Fig. 5, Ala substitution of Ser-245 and -361 resulted in about 50% reduction in VIPR-RP phosphorylation, whereas
Phosphorylation and Transcription Repression

mutations on Ser-189, -338, and Thr-372 had no effect. To confirm that Ser-245/361 are responsible for VIPR-RP phosphorylation by PKA, both of these residues were changed to Ala. As shown in Fig. 6C, substitution of Ser at both positions 245 and 361 resulted in complete loss of phosphorylation. These results indicate that VIPR-RP is phosphorylated by PKA on Ser-245/361.

**Phosphorylation by PKA Inhibits VIPR-RP Transcriptional Repression Function**—To test whether PKA phosphorylation of VIPR-RP affects its transcriptional repression activity, we performed transient transfections in the kinase A-defective F9 teratocarcinoma cells. As shown in Fig. 7, when co-transfected with the wild type VIPR-RP alone, the reporter gene 4FTKLUC activity was reduced 8-fold. Co-transfection with a metallothionein vector expressing the C-subunit (24) of PKA did not have significant effect on 4FTKLUC activity. Co-transfection of the C-subunit together with VIPR-RP caused a 2-fold reduction on 4FTKLUC activity, suggesting that phosphorylation by PKA inhibits VIPR-RP function as a transcriptional repressor. This observation was confirmed by co-transfecting F9 cells with VIPR-RP mutant that was defective in the kinase A phosphorylation motif (S245A/S361A). This mutant alone was able to repress 4FTKLUC activity to the same level as the wild type VIPR-RP (Fig. 7). However, co-transfection with the C-subunit expression vector no longer inhibited the transcriptional repression activity of the mutant VIPR-RP (Fig. 7).

**Effects of Phosphorylation of CK-II and PKA Sites on VIPR-RP Subcellular Localization**—To determine the mechanisms by which phosphorylation of VIPR-RP by CK-II and PKA affects its repression function, we asked whether phosphorylation by these kinases might have an effect on the subcellular distribution of this transcription factor. Toward this end, we transfected COS-7 cells with expression vector containing GFP fused to wild type VIPR-RP or mutants containing defective phosphorylation sites for either CK-II or PKA. The expression of the fusion proteins was examined under the fluorescence microscope. As shown in Fig. 8 (A and B), both the wild type and mutant containing defective phosphorylation sites for PKA were almost exclusively localized to the nucleus (Fig. 6, A and B). However, mutant with defective CK-II phosphorylation sites was either localized to both the nucleus and the cytoplasm, or exclusively in the cytoplasm (Fig. 8C). These results suggest that phosphorylation by PKA does not affect VIPR-RP subcellular localization, whereas phosphorylation of the CK-II sites enhances VIPR-RP nuclear translocation.

**DISCUSSION**

The experiments described in this study represent the initial characterization of VIPR-RP functional domains. Our results showed that amino acids 367–475 are required for specific DNA binding of VIPR-RP to its binding site in the VIPR 5′-flanking region. Although this region does not contain any recognizable consensus for characterized DNA-binding proteins, it contains two predicted α-helical regions of 14 and 13 amino acids, located between amino acids 413 and 426 and between 450 and 462. Identical α-helical regions are also present in DSEB DNA binding domain (7). The importance of these putative helices in DSEB DNA binding was demonstrated by mutagenesis studies (7). An alanine to proline substitution within either helix resulted in complete loss of DNA binding (7). Interestingly, the amino acid sequences required for DNA binding of both VIPR-RP and DSEB also contain the region (amino acids 402–474) where this family of proteins share high identity with bacterial DNA ligases. The DNA binding domains of the DNA ligases have yet to be characterized. However, based on the high degree of homology, it is possible that this region within the ligases may be involved in DNA binding.

Our previous study showed that VIPR-RP is able to repress transcription of a reporter plasmid containing four copies of its binding sequence when co-transfected into COS-7 cells (6). The evidence in this study indicates that VIPR-RP contains two separate transcriptional repression domains, located at the N terminus between amino acids 50 and 101 and C-terminal to the DNA binding domain between positions 469 and 527. Each domain is capable of transcriptional repression by itself when fused to the GAL4 DNA binding domain, if amino acids between 178 and 377 are deleted. However, both domains are required for repression function in the presence of this sequence. Many transcription factors contain multiple activation/repression domains. For example, the glucocorticoid receptor has two activation domains located in the glucocorticoid receptor N terminus and C-terminal of the DNA binding domain (25). Four separate activation domains have been identified in the thyroid receptor. One domain was localized in the N terminus, and the other three were identified in the C terminus adjacent to the ligand binding domain (26).

The activity of many transcription factors is regulated by protein phosphorylation (27). Some transcription factors bind to DNA in unphosphorylated form; however, phosphorylation is required for their transactivation functions. An example of this mechanism is illustrated by cAMP response element-binding protein. Increases in intracellular cAMP levels induce phosphorylation of cAMP response element-binding protein at a serine residue at position 133 by protein kinase A (28), enhancing its ability to activate transcription without affecting its intracellular location or DNA binding (28). A second mechanism involves phosphorylation-induced nuclear translocation of transcription factors that are present in a latent state in the cytoplasm. For example, a single phosphorylation site on STAT1 (signal transducers and activators of transcription), Tyr-701, appears to be both necessary and sufficient to mediate nuclear translocation, DNA binding activity, and transcriptional activation by interferon-γ (29, 30). The presence of consensus phosphorylation sites for PKA and CK-II in VIPR-RP prompted us to ask the question whether VIPR-RP is a substrate for these kinases and what effects phosphorylation by these kinases have on VIPR-RP function. Our results showed...
that VIPR-RP was phosphorylated in vitro by PKA on Ser-245/361 and by CK-II on Ser-69/71 and Thr-110. PKA appears to be the major mediator of cAMP responses in mammalian cells (22). Increased intracellular cAMP levels activates PKA, and induces transport of the C-subunit into the nucleus (31), where it mediates phosphorylation of the transcription factors that activate cAMP-responsive genes. Furthermore, microinjection of the C-subunit into cells can directly activate transcription of cAMP-responsive gene c-fos and the gene for vasoactive intestinal polypeptide (32). Our results showed that phosphorylation by PKA inhibits VIPR-RP transcriptional repression without affecting its subcellular localization. Since no cAMP-responsive element is present in the VIPR1 gene, phosphorylation of VIPR-RP by PKA may provide an indirect mechanism for cAMP-regulated gene expression. Instead of directly acting on the cAMP-responsive element, increased intracellular cAMP levels may activate VIPR 1 gene transcription by inhibition of the activity of its transcriptional repressor, VIPR-RP.

CK-II is a ubiquitous kinase, which has been shown to be localized in both the nucleus and the cytoplasm (33) and can be activated following growth factor stimulation (34, 35). A number of CK-II substrates have been identified. These include proteins involved in regulation of transcription, translation, as well as components of signaling pathways (reviewed in 21). For example, proto-oncogene c-myc was shown to be phosphorylated within the acidic activation domain by CK-II, and it was postulated that CK-II mediated phosphorylation of Myc plays a role in signal transduction of mitogenic stimuli to the nucleus (36). Although we have not directly tested whether VIPR-RP is phosphorylated by CK-II in vivo, we demonstrated that in transfected cells VIPR-RP containing defective CK-II sites was not able to repress transcription of the reporter gene to the same level as the wild type VIPR-RP, suggesting that the activity of VIPR-RP can be modified by phosphorylation of the CK-II sites, either by CK-II itself or by other kinases that recognized the same phosphorylation site.

CK-II phosphorylation sites were found in many nuclear proteins in the vicinity of their nuclear localization signals (NLS), including c-Myc (37, 38), p53 (39, 40), and SV40 T-antigen (41, 42). The average distance between CK-II site and NLS in these proteins is 23 ± 12 amino acids. It has been shown that phosphorylation by CK-II of a serine residue near the NLS of SV40 T-antigen enhanced the rate of nuclear transport of the protein (42). It was postulated that a conformational change of the NLS, induced by CK-II phosphorylation at the nearby site, might modulate the affinity of NLS for its presumptive receptor. The CK-II phosphorylation sites identified in VIPR-RP differ from these nuclear proteins in that they are not localized near the putative NLS. Three putative bipartite NLSs are present between amino acids 480 and 497, 510 and 527, and 523 and 540, whereas the CK-II sites are located in the N terminus. Our results indicated that phosphorylation of the CK-II sites enhances VIPR-RP nuclear localization, because mutant defective in CK-II phosphorylation sites is partially localized in the cytoplasm. It appears likely that phosphorylation of the CK-II sites potentiates the ability of VIPR-RP to repress transcription by ensuring its nuclear localization.

In summary, we have identified functional domains that are important for VIPR-RP DNA binding and transcriptional repression. We have demonstrated that phosphorylation by PKA and on CK-II consensus sites modulates VIPR-RP function through different mechanisms.

Acknowledgments—I thank Drs. A. Banaihmad, V. K. Chatterjee, and G. S. McKnight for plasmids and Dr. R. E. McGehee, Jr. for anti-DSEB antibody.

REFERENCES
1. Pei, L. (1997) Regul. Pept. 71, 153–161
2. Ishihara, T., Shigemoto, R., Mori, K., Takahashi, K., and Nagata, S. (1992) Neuron 8, 811–819
3. Usdin T. B., Bonner, T. I., and Menez, E. (1994) Endocrinology 135, 2662–2680
1182  Phosphorylation and Transcription Repression

4. Pei, L., Melmed, S. (1995) Biochem. J. 308, 719–923
5. Pei, L. (1996) J. Biol. Chem. 271, 20879–20884
6. Pei, L. (1998) J. Biol. Chem. 273, 19902–19908
7. McGhee, R. E., Jr., and Habener, J. F. (1995) Mol. Endocrinol. 9, 487–501
8. Burko, P. K., Utani, A., Pan, Z. Q., and Yamada, Y. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11543–11547
9. Lucklow, B., Bunz, F., Stillman, B., Lichter, P., and Schutz, G. (1998) Mol. Cell. Biol. 14, 1626–1634
10. Lu, Y., Zeff, S., and Riegel, A. T. (1993) Biochem. Biophys. Res. Commun. 193, 779–786
11. Jin, W. D., Boutillier, A.-L., Glucksman, M. J., Salton, S. R. J., Lichter, P., and Schutz, G. (1994) Mol. Cell. Biol. 14, 1626–1634
12. Burbelo, P. K., Utani, A., Pan, Z. Q., and Yamada, Y. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11543–11547
13. Lauer, G., Rudd, E. A., McKay, D. L., Ally, D., and Backman, K. C. (1991) J. Bacteriol. 173, 5047–5053
14. Tsurimoto, T., and Stillman, B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1023–1037
15. Tone, Y., Collingwood, T. N., Adams, M., and Chatterjee, V. K. (1994) J. Biol. Chem. 269, 31157–31161
16. Baniahmad, A., Kohne, A. C., and Renkawitz, R. (1992) EMBO J. 11, 1015–1023
17. Carey, M. F., Kakidani, H., Leatherwood, J., Mostashari, F., and Ptashne, M. (1989) J. Mol. Biol. 209, 425–432
18. Silver, P. A., Chiang, A., and Sadler, I. (1988) Genes Dev. 2, 707–717
19. Meggio, F., Marin, O., and Pinna, L. A. (1994) Cell Mol. Biol. Res. 40, 401–409
20. Pinna, L. A. (1990) Biochim. Biophys. Acta 1054, 267–284
21. Montminy, M. R., Sevino, K. A., Wagner, J. A., Mandel, G., and Goodman R. H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6682–6686
22. Costa, D. B., El-Maghrabi, M. R., and Pilkus, S. J. (1986) J. Biol. Chem. 261, 2987–2993
23. Mellon, P. L., Clegg, C. H., Correll, L. A., and McKnight, G. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4887–4892
24. Hollenberg, S. M., and Evans, R. M. (1988) Cell 55, 899–906
25. Baniahmad, A., Leng, X., Burris, T. P., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1995) Mol. Cell. Biol. 15, 76–86
26. Karin, M., and Hunter, T. (1995) Curr. Biol. 5, 747–757
27. Gonzalez, G. A., and Montminy, M. R. (1989) Cell 59, 475–680
28. Shaulsky, G., Goldfinger, N., Ben-Ze'ev, A., and Rotter, V. (1991) Mol. Cell. Biol. 10, 6565–6577
29. Scheidtman, K.-H., Buck, M., Scheider, J., Kalderon, D., Fanning, E., and Smith, A. E. (1991) J. Virol. 65, 1479–1490
30. Kalderon, D., Richardson, W. D., Markham, A. F., and Smith, A. E. (1984) Nature 308, 109–118
31. Ribas, H.-P., Jans, D. A., Fan, H., and Peters, R. (1991) EMBO J. 10, 633–639