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The N Terminus Domain of Type VI Adenylyl Cyclase Mediates Its Inhibition by Protein Kinase C

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ABSTRACT

Previous results from our laboratory have shown that phosphorylation of type VI adenylyl cyclase (ACVI) by protein kinase C (PKC) caused suppression of adenylyl cyclase activity. In the present study, we investigated the role of the N terminus cytosolic domain of ACVI in this PKC-mediated inhibition of ACVI. Removal of amino acids 1 to 86 of ACVI or mutation of Ser^{10} (a potential PKC phosphorylation site) into alanine significantly relieved the PKC-mediated inhibition and markedly reduced the PKC-evoked protein phosphorylation. PKC also effectively phosphorylated a recombinant N terminus cytosolic domain (amino acids 1–160) protein of ACVI and a synthetic peptide representing Ser^{10}. In addition, the amino acids 1 to 86 truncated mutant exhibited kinetic properties similar to those of the wild type. Taken together, these data demonstrate that the highly variable N terminus cytoplasmic domain of ACVI is a regulatory domain with a critical role in PKC-mediated suppression, which is a hallmark of this adenylyl cyclase isozyme. In addition, Ser^{10} was found to serve as an acceptor for the PKC-mediated phosphorylating transfer of ACVI.

The adenylyl cyclase (AC) family contains a group of enzymes that synthesize cAMP on stimulation (Taussig and Gilman, 1995). To date, at least nine ACs have been isolated and characterized (Tang and Hurley, 1998). Although these enzymes all are activated by the $G_{s\alpha}$ protein, each is under very distinct regulation. We have recently reported that stimulation of the A_{2A} adenosine receptors activated calcium-independent protein kinase C (PKC), which phosphorylated and inhibited type VI AC (ACVI; Lai et al., 1997). Expression of ACVI immunoreactivity was observed in many brain regions (mostly neurons), suggesting that ACVI might participate in the regulation of classical neurotransmitter systems and play a very important role in the central nervous system (Liu et al., 1998).

All of the ACs identified contain two hydrophobic regions that comprise six transmembrane helices and three large cytoplasmic domains (N, C1a/b, and C2). Truncated C1a and C2 domains associate and form a catalytic unit, which is activated by $G_{s\alpha}$ and forskolin (Yan et al., 1996; Dessauer et al., 1997; Scholich et al., 1997b). Structural details of this catalytic unit have recently been revealed by X-ray studies (Yan et al., 1996; Tesmer et al., 1997). In contrast to the well-defined analysis of the C1a/C2 domains, very little is known regarding the role of the NH_2 terminus cytosolic domain (N). We speculate that the N terminus domains of ACV and ACVI, being highly variable and relatively long (239 amino acids and 163 amino acids for ACV and ACVI, respectively; Cooper et al., 1994), may play a role in regulating the two calcium-inhibitable ACs. In the present study, we show that a partial truncation of the N terminus domain of ACVI that removed one potential PKC phosphorylation site (Ser^{10}), as well as mutational inactivation of Ser^{10} into alanine, significantly prevent the inhibitory effect of AC activity by PKC. This result reveals an important regulatory role for a less-studied, highly variable domain of the AC family.

Materials and Methods

Expression of Recombinant ACVI in Sf21 Cells. The cDNA of rat ACVI was kindly provided by Dr. R. Iyengar (Premont et al., 1992). The N terminus-truncated mutants (designated as ΔY155-ACVI and ΔA87-ACVI) and the N terminus domain of ACVI were produced with the polymerase chain reaction (PCR) technique. DNA amplification was carried out with DynaZyme[a] thermostable DNA polymerase (Finnzymes, Espoo, Finland) as described previously (Chang et al., 1997). Nucleotide sequences of the amplified DNA fragments were confirmed by DNA sequencing. Primers for creating the N-truncated ΔY155-ACVI cDNA (encoding the amino acids 155 to 1181 of ACVI) were as follows: 5′-AAAGGATCCAAGCAGTTC-

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ABBREVIATIONS: AC, adenylyl cyclase; ACVI, type VI adenylyl cyclase; dNTP, deoxynucleoside triphosphate; N, NH_2-terminus cytosolic domain; PCR, polymerase chain reaction; PKC, protein kinase C; SSC, standard saline citrate; WT, wild type.

CCGTCCGCC-3′ and 5′-GGTGAATTCTAACTGCTGGGGCCCCCA-3′. Primers for creating the N-truncated ΔA87-ACVI cDNA (encoding amino acids 87 to 1181) were as follows: 5′-AAAGGATCCGCTGGC-CCGGGAAGGGGT-3′ and 5′-GGTGAATTCTAACTGCTGGGGCCCC-CA-3′. The resultant PCR products were subcloned into a baculovirus transfer vector (pVL1393) and expressed in Sf21 cells. For the amplification of the DNA encoding the N terminus domain (amino acids 1–160), 5′-CATATGCCCCTGCCCGTGGCC-3′ and 5′-CGGGATCCGT-TCATCTGGAAGAAGTA-3′ were used in the PCR reaction. The resultant PCR product was ligated to the C terminus of a hexahistidine tag, and then subcloned into pVL1393 for baculoviral expression. Expressions of the wild-type (WT), the N-truncated mutants (ΔY155-ACVI and ΔA87-ACVI), and the N terminus domain of ACVI were carried out in a recombinant baculovirus-driven Sf21 cell system following the protocol of the manufacturer (Pharmingen, San Diego, CA). Membrane fractions were collected as described above from Sf21 cells infected with the desired virus 68 to 72 h after infection.

PCR Mutation. The S10A-ACVI mutant was created by a two-step PCR technique as described previously (Horton et al., 1989) with the following primers: 5′-ATTCATACCGTCCCACCA-3′; 5′-CCGATCCGGTGCTGGGCGCA-3′; 5′-TGCGCCCAGCACCGGATC-GG-3′; 5′-CGGAAGCTATGTCGGTTA-3′; and pVL1393-ACVI as the DNA template. The resultant DNA fragment, which encoded amino acids 1 to 216 of ACVI, contained a single point mutation of S10A. The PCR product was then digested with *Bam*HI and *BssH*II, and subcloned back into the *Bam*HI/*BssH*II-digested pVL1393-ACVI to replace the WT fragment. The mutation of S10A was confirmed by DNA sequencing.

AC Assay. AC activity was assayed as previously described (Chern et al., 1995). Briefly, cells were sonicated with a W-380 sonicator (Ultrasonics Inc., Farmingdale, NY) at a setting of 20% output power for a total of 45 s. The homogenate was centrifuged at 50,000*g* for 30 min to collect the P1 membrane fractions. The AC activity assay was performed at 37°C for 10 min in a 400-μl reaction mixture containing 1 mM ATP, 100 mM NaCl, 0.4 U adenosine deaminase, 50 mM HEPES, 0.5 mM 3-isobutyl-1-methylxanthine, 6 mM $MgCl_2$, 1 μM GTP, and 20 μg of membrane protein. Reactions were stopped by 0.6 ml of 10% trichloroacetic acid (TCA). The cAMP formed was isolated by Dowex chromatography (Sigma Chemical Co., St. Louis, MO) and assayed by radioimmunoassay with the cAMP ^{125}I-assay system (Amersham Int., Little Chalfont, United Kingdom). The ACVI activity was determined as the difference between cyclase activities measured in membrane fractions collected from Sf21 cells infected with the ACVI virus and with the control *Autographa californica* nuclear polyhedrosis virus (AcNPV). The endogenous cyclase activities in Sf21 cells represented approximately 20% of the total activity. The enzyme activity was linear for up to 30 min with membrane proteins up to 40 μg.

The rate of AC was analyzed with the Heriri-Michaelis-Menten equation (Segel, 1976):

$$v = V_{max}/(1 + (K_m/[X])),$$

in which v is the velocity, V_{max} is the maximal velocity, X is the indicated reagent (ATP, $G_{s\alpha}$, or forskolin), and K_m is the Michaelis-Menten constant. The kinetic parameters, V_{max} and K_m, were obtained by fitting the data with the above equation with the SigmaPlot nonlinear curve-fitting and plotting software (SigmaPlot 4.0; SPSS Inc., Chicago, IL).

Recombinant $G_{s\alpha}$ Protein Expression. The expression construct (pQE60/H6-$G_{s\alpha}$) of $G_{s\alpha}$ protein was a generous gift from Dr. W.-J. Tang (University of Chicago, Chicago, IL). The hexahistidine-tagged $G_{s\alpha}$ protein was expressed in *Escherichia coli* and purified with the His · Bind metal chelation resin (Novagen Inc., Madison, WI) as described elsewhere (Yan et al., 1998). To stimulate AC activity, the indicated concentration of $G_{s\alpha}$ protein was activated by GTPγs (20 μM) in a 100-μl reaction mixture containing 10 mM $MgCl_2$, 20 mM Tris, 1 mM EDTA, and 1 mM dithiothreitol for 20 min at 20°C.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting. Membrane fractions were separated on 8% separating gels according to the method of Laemmli (Laemmli, 1970; Lai et al., 1997). After electrophoresis, the gel was transferred to a polyvinylidene fluoride membrane, blocked with 5% skim milk in PBS (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4), then incubated with the desired antiserum at 4°C overnight. The polyclonal antibodies AC6N and AC6D, were raised against amino acids 1 to 19 (the N terminus) and amino acids 987 to 1187 (the C2 domain) of ACVI (Lai et al., 1997; Liu et al., 1998). Typically, 1:500 and 1:5000 dilutions were used for AC6N and AC6D, respectively, unless stated otherwise. The membranes were incubated with peroxidase-conjugated donkey anti-rabbit IgG (1:5000 dilution; Amersham) for 1 h at room temperature, and washed with PBS containing 0.1% Tween-20. The immunoreactive bands were stained with a light-emitting nonradioactive method (Amersham).

Immunoprecipitation and In Vitro Phosphorylation. To carry out the protein phosphorylation study, the WT or the mutant of ACVI expressed in Sf21 cells was purified by immunoprecipitation with AC6D as described previously (Lai et al., 1997). The recombinant N terminus domain of ACVI was purified by immunoprecipitation with AC6N. Immunocomplexes were purified with Sephadex conjugated protein A (Sigma) and then washed three times with ice-cold RIPA buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS 10 μM PMSF, 100 nM leupeptin, and 50 mM Tris, pH 8.0). Phosphorylation by PKC was carried out in a final volume of a 0.1-ml reaction mixture containing 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25% BSA, 10 μM H89, 0.5 mM [γ-^{32}P]ATP (2 Ci/mol), 0.1 mM leupeptin, 40 μM phenylmethylsulfonyl fluoride, 30 nM okadic acid, 0.2 mM Na vanadate, and 20 mM Tris (pH 7.5). The phosphorylation reaction was initiated by the addition of PKC (0.1 mU) purified from rat brain (Boehringer Mannheim, Indianapolis, IN) for 30 min at 4°C, and terminated by the addition of 2× SDS sample treatment buffer. The samples were then boiled for 5 min and analyzed by SDS-PAGE (8%) and Western blot. To visualize the phosphorylation of ACVI by PKC, immunoblots were rinsed twice with PBS, air dried, and autoradiographed.

Peptide Phosphorylation. Peptides were synthesized and purchased from Genosys (Woodlands, TX). The composition and amount of each peptide were further confirmed by amino acid analysis (Analytical Biotechnology Services, National Taiwan University, Taipei, Taiwan). Names and sequences of the peptides are as follows: S10, PVARSGSGRSSMS (amino acids 4–16 of ACVI); S123, EVAP-DTSPRSGPS (amino acids 117–129 of ACVI); and S145, QSKQFP-SAKLERL (amino acids 139–151 of ACVI). The well-characterized peptide substrate of PKC MBP4–14 (QKRPSQRSKTL; Yasuda et al., 1990) was obtained from Sigma. Phosphorylation of each peptide was carried out in a final volume of a 25-μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mg/ml phosphatidylserine, 20 μg/ml diolein, 25 μM [γ-^{32}P]ATP (2 μCi/nmol), the desired peptide, and 0.01 mU of PKC. After a 30-min incubation at 30°C, the reaction was terminated by boiling for 5 min. The phosphorylated peptides were segregated from [γ-^{32}P]ATP by 20% acrylamide SDS-PAGE as described previously (Honegger et al., 1988). The gels were fixed with two changes of 30% (v/v) methanol followed by two changes of 10% (v/v) acetic acid and exposed to film at −80°C. For some of the experiments, the incorporation of [^{32}P]phosphate into the peptide was determined by scintillation counting of phosphorylated peptide excised from the gel.

Results and Discussion

It is well known that each isozyme in the AC superfamily is under very distinct regulation. For example, ACVI is inhibited by physiologically relevant concentrations of Ca^{2+}, whereas other AC isozymes (ACI and ACIII) are activated by Ca^{2+} in the presence of calmodulin (Cooper et al., 1994). In

addition, although PKC has been implicated in stimulation of ACII and ACV (Choi et al., 1993; Jacobowitz et al., 1994; Kawabe et al., 1994), it markedly inhibits the activity of ACVI (Lai et al., 1997). These observations suggest the existence of isozyme-specific structural components that can interact directly or indirectly with the conserved catalytic core to modulate AC functions. To identify a possible regulating component, we analyzed the topology of ACVI with VHMPT (Fig. 1). VHMPT is an automated membrane protein topology generator with the capability of displaying evolutionarily conserved (and varied) domains, and is particularly useful for analyzing evolutionarily conserved gene families (Lin and Hwang, 1998). As shown in Fig. 1, the highly conserved C1a and C2 catalytic core domain of the AC family is easily visible in a topological image created with the VHMPT program. Whereas conserved residues/domains are often of primary interest in studying a homologous enzyme family like AC, highly variable regions are likely to confer functional subtleties and specificities for different members of the family. The present study targeted the latter. Except for a few residues near its C-terminal end, the N-terminal domain is visualized to be highly variable (Fig. 1), and therefore representing a good target for investigation.

We previously showed that PKC phosphorylated and inhibited ACVI (Lai et al., 1997). To investigate whether the highly variable N terminus domain is involved in PKC-mediated suppression of ACVI activity, two N terminus deletion mutants that lack the whole N terminus domain (amino acids 1 to 154; designated as ΔY155-ACVI) or the N terminus 1 to 86 amino acids (designated as ΔA87-ACVI) were created with the PCR technique. Recombinant ΔY155-ACVI protein was not detectable with the baculoviral expression system, suggesting that the N terminus domain of ACVI is important for its protein stability (data not shown). In contrast, recombinant ΔA87-ACVI protein was readily produced as shown in Fig. 2A. For both the WT and the N-truncated ΔA87-ACVI mutant, two immunoreactive bands were observed. Blocking glycosylation with tunicamycin (1 mg/ml) effectively removed the higher ACVI-immunoreactive band (data not shown), suggesting that ACVI is partially glycosylated in Sf21 cells. As expected, ΔA87-ACVI showed a slightly faster mobility in SDS-PAGE compared with that of WT ACVI (Fig. 2A). Most importantly, phosphorylation of ΔA87-ACVI by PKC was significantly lower than that of the WT ACVI (Fig. 2, A and C). Analysis of the ACVI sequence with the Genetics Computer Group program (Madison, WI) revealed a potential PKC phosphorylation site, Ser^{10}, in the truncated N terminus (amino acids 1–86). By using a PCR-based mutagenesis method, we mutated Ser^{10} into alanine to determine whether the lack of Ser^{10} is responsible for the reduction of PKC-evoked phosphorylation of ΔA87-ACVI. As demonstrated in Fig. 2, B and C, phosphorylation of the single-point mutation

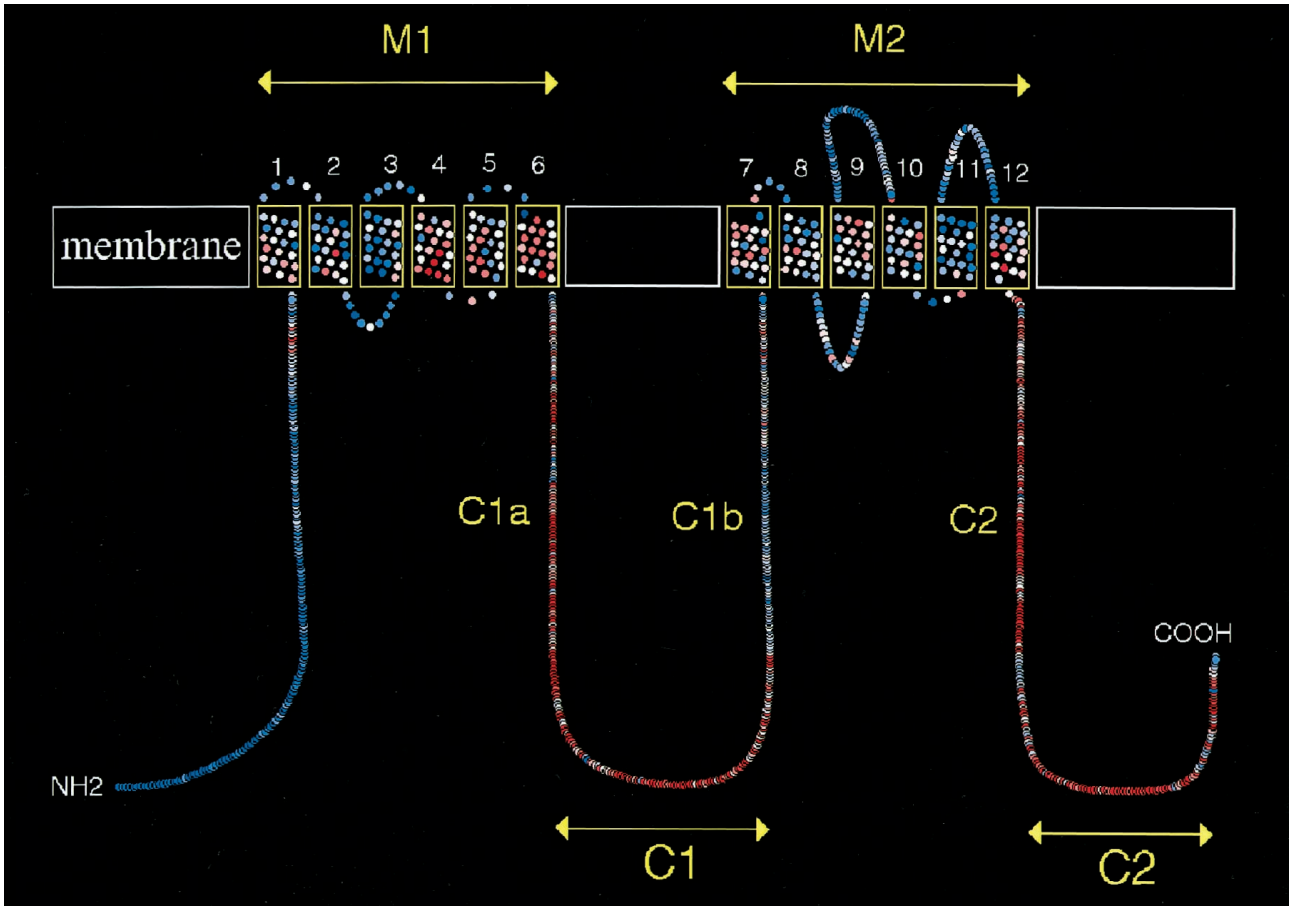

Fig. 1. A putative transmembrane topology of ACVI. This ACVI topology was produced with the program, VHMPT, an automated graphical viewer and editor for membrane protein topologies (Lin and Hwang, 1998). Each circle represents an amino acid color-coded by its normalized conservation score in the alignment of nine AC sequences. "Blue ← white ← red" corresponds to score "0 ← 0.5 ← 1", with 1 being totally conserved. Sequences of type I to type IX ACs are derived from Genebank (access numbers M25579. Gb_Om, M80550. Gb_Ro, M55075. Gb_Ro, M80633. Gb_Ro, M96159. Gb_Ro, M96160. Gb_Ro, U12919. Gb_Ro, L26986. Gb_Ro, U30602. Gb_Ro, respectively).

S10A-ACVI by PKC was also reduced compared with that of the WT.

We next examined whether the N terminus domain itself is phosphorylated by PKC. As shown in Fig. 3, the recombinant N terminus domain (amino acids 1–160) of ACVI migrated as a protein of 28 kDa and was effectively phosphorylated by PKC. Because synthetic peptides representing the potential phosphorylation sites have been used to assess the most likely phosphorylation site (Luscher et al., 1990; Graff et al., 1991), we synthesized peptides based on the three potential PKC phosphorylation sites (Ser^{10}, Ser^{123}, and Ser^{145}) of ACVI located in its N terminus domain, and tested whether these peptides serve as substrates for PKC. As shown in Fig. 4A, PKC phosphorylated only the S10 peptide, not the S123 or S145 peptide. Phosphorylation of the S10 peptide by PKC was linear up to 60 min, whereas no significant phosphorylation of the S123 peptide or the S145 peptide was detected (Fig. 4B).

We then determined the effect of PKC treatment on the enzymatic properties of the WT ACVI and the two mutant ACVI variants. Membranes prepared from Sf21 cells infected with the indicated ACVI baculovirus were incubated with purified PKC, and then assayed for enzymatic properties. As shown in Table 1, the V_{max} value of the PKC-treated WT ACVI was significantly lower than those of the nontreated enzyme by approximately 70%. It is also noteworthy that the treatment of PKC did not significantly alter the K_m for substrate, nor did it markedly affect the EC_{50} for forskolin. The affinity of ACVI toward the $G_{s\alpha}$ protein might be reduced by PKC because the EC_{50} value of $G_{s\alpha}$ for PKC-treated ACVI was almost twice that of the control group (nontreated). However, this difference in the $G_{s\alpha}$ protein affinity by PKC treatment was not statistically significant ($t_{5,28} = 1.936$ and $p > .05$ when comparing the nontreated and PKC-treated groups).

Mutational inactivation of Ser^{10} significantly reduced PKC-mediated inhibition ($t_{5,18} = 3.48$ and $p < .05$ when comparing the relative activities of the PKC-treated WT and the PKC-treated S10A-ACVI groups). Reduced PKC-evoked inhibition in the catalytic activity of S10A-ACVI correlated well with the decreased phosphorylation levels of S10A-ACVI compared with that of the WT, demonstrating that Ser^{10} is critical for PKC-mediated inhibition of ACVI activity. Nevertheless, mutation of Ser^{10} only partially relieved PKC-mediated inhibition, further implying that there are other residues, in addition to Ser^{10}, that might play an important role in the phosphorylation and suppression of ACVI by PKC.

Truncation of amino acids 1–86 also effectively reduced PKC-mediated inhibition ($t_{5,18} = 5.7$ and $p < .05$ when comparing the relative activities of the PKC-treated WT and the PKC-treated ΔA87-ACVI groups), supporting the hypothesis that Ser^{10} is important for PKC-mediated inhibition of ACVI. For reasons currently unknown, removal of the first 86 amino acids appears to be more effective than site-directed mutation of Ser^{10} in relieving the inhibition of ACVI activity by PKC. As shown in Table 1, PKC treatment did not exert a statistically significant effect on V_{max} of the ΔA87-ACVI protein ($t_{5,18} = 2.49$ and $p > .05$ when comparing the relative

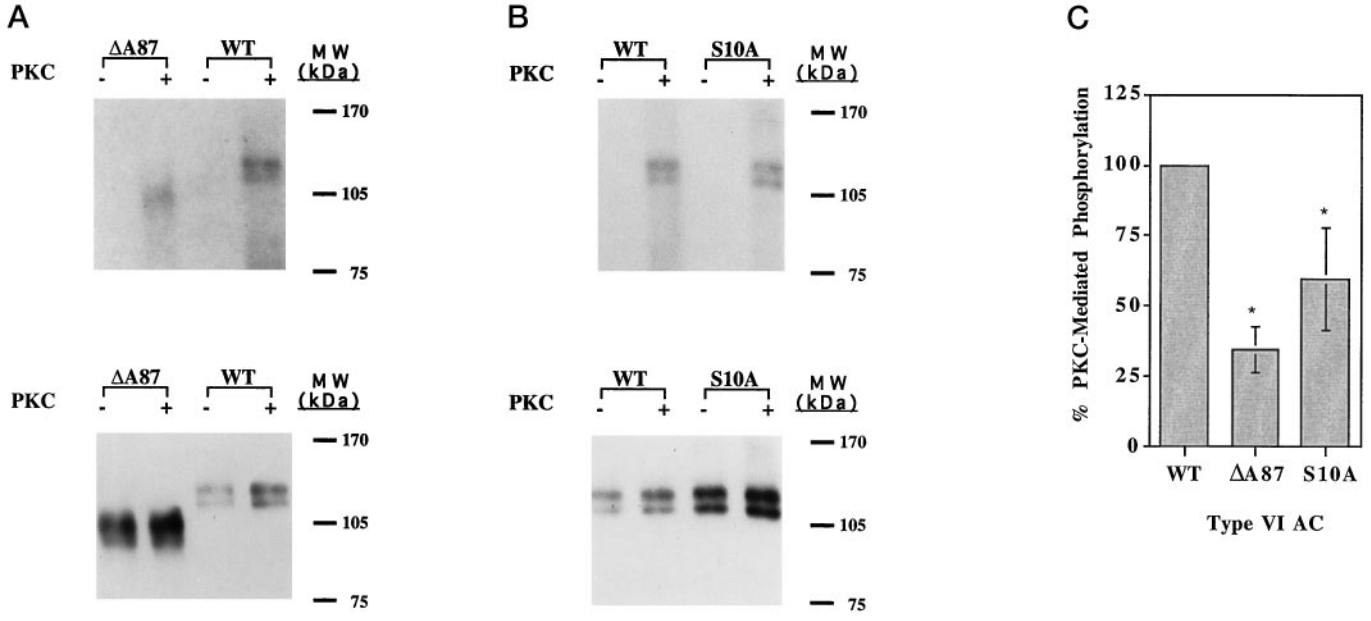

Fig. 2. Truncation of the first 86 amino acids of ACVI or mutation of Ser^{10} into alanine significantly reduced the PKC-mediated phosphorylation of ACVI. Recombinant WT ACVI and two ACVI mutants (ΔA87-ACVI, and S10A-ACVI) were prepared in Sf21 cells and purified with AC6D as described in the text. Purified WT or mutant ACVI protein was then incubated with or without PKC (0.1 mU) as indicated for 30 min at 4°C in the presence of [γ-^{32}P]ATP. At the end of incubation, the samples were boiled for 5 min and analyzed by Western blot analysis (lower panel) with AC6D (1:5000 dilution) to indicate the amount of ACVI protein present in each sample. The same blot was then analyzed by autoradiography (upper panel) to determine the PKC-mediated phosphorylation levels. To clearly visualize the phosphorylation of ΔA87-ACVI (A) and S10A-ACVI (B), approximately 2- to 3-fold higher amounts of the ACVI mutant proteins than the amount of WT ACVI protein were loaded in the gels. C, values for phosphorylation extent of the PKC-treated ACVI variant (i.e., integrated absorbance units of phosphorylation signal ÷ protein signal of the indicated protein) are expressed as percentages of the phosphorylation of the PKC-treated WT protein. The data were generated by quantitative computing densitometry of autoradiograms from three independent experiments with the image analysis software package, ImageQuant v.3.15 (Molecular Dynamics, Sunnyvale, CA). Determination of the phosphorylation levels and the protein levels of the PKC-treated ACVI variants were performed with relatively short-exposure films. Because no phosphorylation signal was detected in the absence of PKC, we did not determine the phosphorylation levels for the control (nontreated) ACVI proteins.

activities of the nontreated and PKC-treated ΔA87-ACVI groups).

As demonstrated in Table 1, the activity (V_{max}) of ACVI was not significantly altered by partial truncation of its N-terminus domain or by the mutational inactivation of Ser^{10} into alanine [$t_{5,16} = 1.71$ and $p > .05$ when comparing the WT (nontreated) with ΔA87-ACVI (nontreated), and $t_{5,16} = 1.49$ and $p > .05$ when comparing the WT (nontreated) with S10A-ACVI (nontreated), respectively]. In addition, kinetic analyses showed that removal of the first 86 amino acids of ACVI did not affect its affinities for ATP. Nor were the EC_{50} values for forskolin and $G_{s\alpha}$ protein affected (Table 1). The observation that ΔA87-ACVI displayed all of these catalytic properties similar to those of the WT enzyme suggests that amino acids 1–86 of ACVI might not significantly contribute to the intrinsic catalytic function carried out by the conserved catalytic core complex of the C1a and C2 domains.

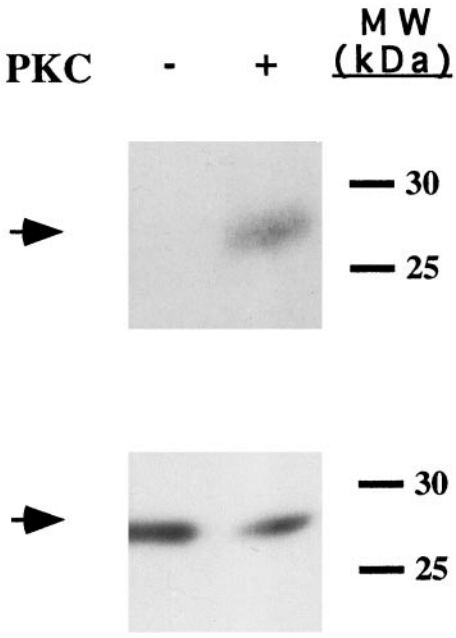

Fig. 3. PKC effectively phosphorylated the recombinant N terminus domain of ACVI. The recombinant N terminus domain of ACVI was produced in Sf21 cells and purified immunologically with AC6N as described in the text. Purified recombinant N-domain protein was then incubated with or without PKC (0.1 mU) as indicated for 30 min at 4°C in the presence of [γ-^{32}P] ATP. At the end of the incubation, the samples were boiled for 5 min and analyzed by Western blot analysis (bottom) with AC6N (1:500 dilution) and by autoradiography (top).

Protein phosphorylation is a very important regulatory mechanism for ACs (Wei et al., 1996; Chen et al., 1997; Lai et al., 1997; Tang and Hurley, 1998). Most of the reported phosphorylation residues of ACs are located in the C1 or C2 domain. For example, by using chemical and immunochemical methods, Bol et al. (1997) reported that Ser^{871} and Thr^{1057} are the two most likely residues for PKC-mediated phosphorylation of ACII. Both residues are located in or very close to the C2 domain. Wei et al. (1996) found that Ser^{1076} of ACIII, which is located in a highly conserved region in the C2 domain of all ACs, appears to be the phosphorylation site by the calmodulin-dependent protein kinase II. Phosphorylation of amino acid residue(s) in the C1b region of ACs also was reported. Wayman et al. (1996) found that the most likely phosphorylation residues of ACI by the calcium/calmodulin-dependent protein kinase IV are Ser^{545} and Ser^{552} in the C1b region. Chen et al. (1997) demonstrated that Ser^{674} in the C1b region of ACVI is apparently the site for PKA phosphor-

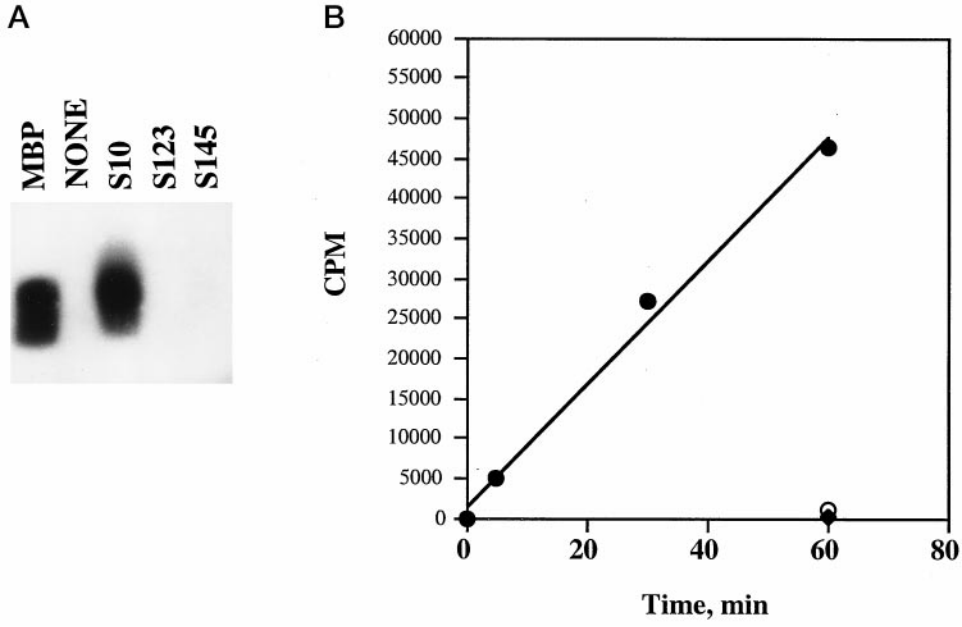

Fig. 4. PKC effectively phosphorylated the S10 peptide of ACVI. A, S10 peptide (1 mM), S123 peptide (1 mM), S145 peptide (1 mM), or MBP peptide (200 μM) was incubated with PKC (0.01 mU) as indicated for 30 min at 30°C in the presence of [γ-^{32}P]ATP (25 μM). At the end of incubation, samples were boiled for 5 min and analyzed by 20% acrylamide SDS-PAGE followed by autoradiography. MBP (MBP4–14) is a well-characterized peptide substrate of PKC (Yasuda et al., 1990). B, phosphorylation of the S10 peptide (●), S123 peptide (♦), and S145 peptide (○) were incubated with PKC (0.01mU) for the indicated period of time at 30°C in the presence of [γ-^{32}P]ATP (25 μM). At the end of incubation, samples were boiled for 5 min, analyzed by 20% acylamide SDS-PAGE followed by autoradiography. Incorporation of 32P-phosphate into the peptide was determined by scintillation counting of the phosphorylated peptide excised from the gel. Each data point represents the average of two determinations. The results are from one representative experiment out of three independent experiments performed.

ylation, which may hinder stimulation of ACVI activity by $G_{s\alpha}$ protein. Our data in the present study demonstrate that the N terminus of ACVI is phosphorylated by PKC and this phosphorylation is involved in the regulation of enzyme activity. It is noteworthy that the first 86 amino acids constitute the most variable cytosolic region of ACVI as compared with other ACs (Fig. 1). Most of the other types of AC have a much shorter N terminus domain (Cooper et al., 1994). Moreover, no similarity in the amino acid sequences was found in most of the N terminus regions, even to another Ca^{2+}-inhibitable AC (ACV) that can be potentiated by PKC (Kawabe et al., 1994). Therefore, this unique region of ACVI may underlie a specificity in its functional regulation. The correlation between a unique motif (domain) and a unique regulation has a precedent example in ACVI. Chen et al. (1997) reported that the PKA-mediated inhibition of $G_{s\alpha}$- stimulated cyclase activity of ACVI is attributable to the presence of a unique PKA-phosphorylation site. In comparison, ACI and ACII, lacking this PKA site, are free of such PKA-mediated inhibition (Chen et al., 1997).

The rat ACVI cDNA used in the present study was isolated from a rat liver cDNA library by Premont et al. (1992). Three additional ACVI cDNA clones have been identified from a canine cardiac cDNA library (Katsushika et al., 1992), a rat hepatoma cell line cDNA library (Krupinski et al., 1992), and a mouse cell line NCB-20 cDNA library (Yoshimura and Cooper, 1992). It is important to note that the rat ACVI reported by Premont et al. (1992) is 14 amino acids longer on the N terminus than on those of the other three ACVI proteins, all of which consequently lack the Ser^{10} PKC-phosphorylation site. Because multiple messages exist for ACVI in several tissues (Premont et al., 1992), such differences in the length of the N terminus of ACVI might result from alternative splicing of the rat ACVI gene. Alternative splicing of a cAMP-specific phosphodiesterase gene (ratPDE3) results in multiple transcripts encoding proteins with divergent N-terminal regions (Monaco et al., 1994), and these splice variants of ratPDE3 are differentially regulated by PKA-dependent phosphorylation (Sette et al., 1994). Splice variants of ACIII and ACVIII have also been demonstrated (Cali et al., 1996; Gautier-Courteille et al., 1998). It remains to be determined, however, whether N terminus-mediated suppression of ACVI by PKC is specific only to the ACVI variant reported by Premont et al. (1992). In addition, it is of great interest to determine the tissue distribution of the ACVI variants with diverse N-terminal regions that might confer differential regulatory modes of ACVI variants by PKC.

Currently, information regarding the role of the N terminus domain of ACs is very limited. Tang et al. (1991) reported that truncation of the first 52 amino acid residues of ACI significantly suppressed its catalytic activity, implying that this domain might be involved in the secondary structure or membrane orientation. Scholich et al. (1997a) found that synergistic stimulation of ACV by $G_{s\alpha}$ and forskolin was dramatically enhanced in the absence of the N terminus and the transmembrane regions. In the absence of amino acids 1 to 86 of ACVI, we demonstrated in the present study that bindings of ATP, forskolin, and $G_{s\alpha}$ to the catalytic domains were not significantly affected, whereas PKC-mediated inhibition of catalytic activity was significantly relieved. It is possible that a ligand binding-induced conformational change is required for catalysis, and this conformational change is hindered by the PKC-phosphorylated N terminus domain, resulting in reduced activity in PKC-treated ACVI. Alternatively, a conformational change within the N terminus, triggered by PKC phosphorylation, may present a barrier to hinder substrate entry or product release, thereby reducing the catalytic rate without significantly altering the structure of the catalytic domain. This latter mechanism would be somewhat analogous to the "ball-and-chain" model of ion channels (Bennilla et al., 1977).

In summary, our study suggests that the N terminus cytosolic domain of ACVI plays a very critical role in the suppression of ACVI activity by PKC, and Ser^{10} is the most likely site of phosphorylation. Our results provide the first clear evidence to indicate that the highly variable N terminus domain of ACs significantly contributes to the regulatory diversity of the AC family.

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TABLE 1

Truncation of the first 86 amino acids of ACVI or mutation of Ser^{10} into alanine significantly relieved PKC-mediated inhibition of the cyclase activity of ACVI

The plasma membrane fractions collected from Sf21 cells infected with the WT, ΔA87-ACVI, or S10A-ACVI were incubated with or without purified PKC and assayed for AC activity. Values represent the mean ± S.E.M. (3 ~ 10 independent experiments). K_m of the substrate (ATP) was determined in the presence of forskolin (5 μM). V_{max} of the forskolin-evoked AC activity and EC_{50} values of $G_{s\alpha}$ and forskolin were measured in the presence of 6 mM $MgCl_2$ and 1 mM ATP. The relative activity of the PKC-treated ACVI variant is expressed as the percentage of the control (nontreated) ACVI variant as shown in parentheses. Statistical significance was evaluated by one-way ANOVA.

ACVI	PKC	K_m	$G_{s\alpha}$, EC_{50}	Forskolin, EC_{50}	V_{max}
		μM	*nM*	*μM*	*pmol/mg/min*
WT	−	133 ± 18	30.4 ± 7.8	8.6 ± 4.8	802.0 ± 211.0 (100% ± 26%)
WT	+	123 ± 15	57.7 ± 16.3	5.2 ± 2.7	244.0 ± 88 (30% ± 11%)*
ΔA87	−	183 ± 11	24.8 ± 5.2	7.1 ± 1.1	1218.9 ± 368.1 (100% ± 30%)
ΔA87	+	153 ± 29	23.8 ± 8.3	9.5 ± 5.5	859.9 ± 201.3 (70% ± 17%)**
S10A	−	207 ± 29	57.1 ± 13.0	18.8 ± 7.2	943.0 ± 225.3 (100% ± 20%)
S10A	+	193 ± 15	43.4 ± 14.0	11.9 ± 4.7	550.2 ± 88.1 (57% ± 6%)*,**

*$p < 0.5$. Specific comparisons between the PKC-treated and the control group of each ACVI variant were performed using the Dunnett method (Winer, 1971). **$p < .05$. Specific comparisons of the relative activity of the PKC-treated groups between the WT and indicated mutant was performed using the Dunnett method.

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