We show herein that removal of the first 86 amino acids (aa) of the N terminus (designated N) of type VI adenylyl cyclase (ACVI) caused the resultant ACVI mutant (ACVI-ΔAA87) to be more greatly inhibited by a Gαi-coupled receptor or activated Gαs protein. Moreover, in vitro binding of the full-length N and C1a domain (designated C1a), which interacts with Gαi, was detected. A truncated N terminus (aa 1–86) also interacted with C1a, suggesting that the C1a-interacting region is located within aa 1–86. Mutation analyses further revealed that N might interact with C1a in the region (aa 434–505) where Gαi is bound. Mutations of two residues (Leu-472 and Val-476) located in this N-binding region of C1a suppressed the interaction between recombinant N and C1a and markedly reduced Gαi-mediated inhibition of ACVI-ΔAA87. Further biochemical analyses of the effect of internal mutations of Leu-472/Val-476 on Gαi-mediated inhibition of wild-type ACVI and ACVI-ΔAA87 suggested that N modulates the Gαi-mediated inhibition of ACVI via binding to C1a when the level of Gαi is low (i.e. around the IC50 value) and that a more complicated interfering mode results when the level of Gαi is high (i.e. ~10- to 20-fold of the IC50 value). Collectively, data presented herein suggest a novel function of the N terminus of ACVI in Gαi-mediated regulation.

The mammalian adenylyl cyclase (AC) superfamily consists of nine membrane-bound isoforms. All of these possess three large cytosolic domains (designated N, C1a, and C2 domains; Fig. 1A), which are separated by two sets of six-transmembrane domains (1–5). The C1a and C2 domains among the nine AC members are highly homologous (with 50–90% similarity in amino acids). In addition, the C1a and C2 domains of each AC share ~50% similarity and form the catalytic core complex, which can be stimulated by forskolin or Gαs proteins (4–6). Crystallographic analysis of the catalytic complex consisting of the C1a domain, the C2 domain, and the GTPγS-bound Gαs protein revealed that forskolin and/or Gαs stimulate ACs by enhancing the interaction between the C1a and C2 domains and by stabilizing the C1a-C2 catalytic core complex (7).

Although most ACs can be activated by Gαs and forskolin, regulation of each AC isozyme differs. Studies of the more-variable N and C1b domains reveal that these two domains may play important regulatory roles. For example, the C1b domain has been implicated in the regulation of AC isozymes mediated by Ca2+/calmodulin, calcineurin, or protein kinase A (8–11). In addition, the C1b domain of ACV and ACVI has been shown to modulate Gαi-activated activation by interacting with catalytic core complexes (12, 13). Others and we have shown that another variable region, the N-terminal domain, also significantly contributes to the regulation of AC activity (14, 15). Specifically, the N-terminal domain of ACVI (amino acids 1–160) plays an important role in the protein kinase C (PKC)-mediated inhibition and phosphorylation of ACVI (14). Removal of the first 86 amino acids (aa) of ACVI reduced the inhibitory effect of PKC on ACVI activity without affecting the basic enzymatic properties, including the affinities toward its substrates and two stimuli (forskolin and Gαs protein (14)). The N-terminal domain of ACVI therefore functions as a regulatory domain. Further biochemical analyses revealed that at least four PKC phosphorylation sites (Ser-10, Ser-568, Ser-674, and Thr-931), located in the three large cytosolic domains of ACVI, significantly contribute to PKC-mediated inhibition of ACVI (16). Intramolecular interactions among the N, C1a/b, and C2 domains of ACVI therefore appear to be important for regulation of ACVI activity.

Gαi-mediated inhibition is a major regulatory feature of the AC superfamily. Among AC members, only ACI, ACV, and ACVI can effectively be inhibited by Gαi proteins (17, 18). Based on results obtained from an in vitro binding assay, the C1a domain was shown to bind myristoylated Gαi proteins and form stable complexes (19, 20). Mutagenesis of full-length ACV in the α2 and α3 helices of the C1a domain revealed several residues important for the inhibition by Gαi proteins. It was postulated that Gαi may exert its inhibitory effect through binding to the C1a domain at the site just opposite the Gαs-binding site on the C2 domain and, subsequently, causes reduced interaction between the C1 and C2 domains (21). In the present study, we present evidence to suggest that the N-terminal domain of ACVI directly interacts with the C1a domain and may play an important role in the regulation of ACVI by Gαi proteins.
Supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 

The homogenate was centrifuged at 50,000 

Briefly, cells were sonicated using a W-380 sonicator (Ul-

Using a light-emitting non-radioactive method (ECL, Amersham Biosciences) for 1 h at room temperature and dark gray is shown in circles B.

HER251 residues (Leu-472 and Val-476) located in the C1a domain, which are involved in the binding of the N terminus and G 

To determine if the recombinant G 

Typically, a 1:5000 dilution was used for AC6D unless stated otherwise. AC6D, was raised against aa 987 – 1187 (the C2 domain) of ACVI (27).

For Western blot analyses, gels were transferred to polyvinylidene difluoride membranes following electrophoresis that were blocked with (26). For Western blot analyses, gels were transferred to polyvinylidene difluoride membranes following electrophoresis that were blocked with (26).

The expression construct of pET11d vector (Novagen). The G 

The expression construct of pET11d vector (Novagen). The G 

Historically, human G proteins were myristoylated, 9% SDS-PAGE gels containing 4 x urea were employed (28) as were Western blot analyses using an anti-G 

The expression construct of pET11d vector (Novagen). The G 

PCR Mutagenesis and Plasmid Construction—Different lengths of

Fig. 1. Type VI adenylyl cyclase (ACVI). A, a schematic presentation of rat ACVI (amino acid sequence derived from M86160.1b). Two residues (Leu-472 and Val-476) located in the C1a domain, which are involved in the binding of the N terminus and Gα, are indicated as open circles. B, a computer model of the VIC1a/VIC2 complex. The model was predicted based on the x-ray complex structure of VCIa and HC2 (7). VIC2 is shown in dark gray. The N-interacting peptide (aa 424–505) of VIC1a is shown in black. The region of VIC1a, which is not involved in the interaction with the N terminus of ACVI, is shown in light gray. Residues located on the α2 (Glu-411, Met-414, and Thr-415) and α3 (Leu-472 and Val-476) helices of C1a, which might be involved in the interaction with Gα, based on a previous mutational analysis of ACVI (19), are indicated.
DNA fragments encoding the C1α and N domains of ACVI were produced using the PCR technique. DNA amplification was carried out in a solution containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 0.2 mg of the desired primers, 0.2 mM of each deoxynucleoside triphosphate (dNTP), a DNA template, and 2 units of Dynazyme™

bound on the Ni-NTA His

®

/TOPO vector using a TA expression kit (Invitrogen; C1α-1, C1α-3, C1α-4, and C1α-5). Nucleotide sequences in all constructs used were confirmed by DNA sequencing.

The L472A/V476A-C1α mutant was created by a two-step PCR technique as described previously (33) with the following primers: 5'-CATATGATGAGAATGGAGATG-3'; 5'-GGATACCTCCTTCTCCCTTTTT-3'; 5'-GGCTCTACGACCGCCGAGATGTCGATCAT-3'; and 5'-GGGGTGCTGTGGACCGCTGTAATAATGGAAC-3', with pVL1393-ACVI as the DNA template. The resultant DNA fragment, which encoded aa 364–575 of ACVI, contained double point mutations at L472A and V476A. The PCR product was then subcloned into the pcDNA3.1/V5-His/TOPO vector using a TA expression kit (Invitrogen; C1α-1, C1α-3, C1α-4, and C1α-5). Nucleotide sequences in all constructs used were confirmed by DNA sequencing. The resultant construct was digested with SacI and Bsu36I and subcloned into the SacI/Bsu36I-digested pVL1393-ACVI construct to create the pVL1393-ACVI-L472A/V476A mutant construct. The eukaryotic expression construct (pDNA3-ACVI-L472A/V476A) of the ACVI-L472A/V476A mutant protein was constructed by subcloning a BamHI/EcoRI fragment encoding ACVI-L472A/V476A from pVL1393-ACVI-L472A/V476A into the corresponding sites of pcDNA3. The ACVI-L472A/V476A mutant protein was created by subcloning the NheI/Bsu36I-digested pDNA3-ACVI-L472A/V476A fragment containing the mutated residues (L472A and V476A) into the NheI/Bsu36I-digested pDNA3-ACVI-L472A/V476A construct.

The L472A/V476A-C1α mutant was created by the two-step PCR technique using two AC-specific primers (5'-GGATACCTAGTCGAGGCTCCTAAAAGGC-3' and 5'-GGCTCTACGACCGCCGAGATGTCGATCAT-3') and pVL1393-ACVI (23) as the DNA template and two AC-specific primers (5'-AACAGAGCAGCCTACGGCT-3' and 5'-GGGGTGCTGTGGACCGCTGTAATAATGGAAC-3') with pVL1393-ACVI as the DNA template. The resultant construct was digested with SacI and Bsu36I and subcloned into the SacI/Bsu36I-digested pVL1393-ACVI construct to create the pVL1393-ACVI-L472A/V476A mutant construct. The eukaryotic expression construct (pDNA3-ACVI-L472A/V476A) of the ACVI-L472A/V476A mutant protein was constructed by subcloning a BamHI/EcoRI fragment encoding ACVI-L472A/V476A from pVL1393-ACVI-L472A/V476A into the corresponding sites of pcDNA3. The ACVI-L472A/V476A mutant construct was digested with SacI and Bsu36I and subcloned into the SacI/Bsu36I-digested pVL1393-ACVI construct to create the pVL1393-ACVI-L472A/V476A mutant construct. The eukaryotic expression construct (pDNA3-ACVI-L472A/V476A) of the ACVI-L472A/V476A mutant protein was constructed by subcloning the NheI/Bsu36I-digested pDNA3-ACVI-L472A/V476A fragment containing the mutated residues (L472A and V476A) into the NheI/Bsu36I-digested pDNA3-ACVI-L472A/V476A construct.

Results

One important regulation for ACVI, which can be observed for only two other AC isozymes (ACI and ACV), is the inhibition mediated by Gαi proteins (17, 18). Because the N-terminal domain is highly variable among AC isozymes, we set out to investigate the role of the N-terminal domain of ACVI in Gαi-mediated inhibition. The wild-type ACVI or an N terminus-truncated ACVI mutant, which lacks the first 1–86 aa and exhibits a smaller size in the SDS-PAGE/Western blot analysis (ACVI-ΔA87; Fig. 2A (14)), was co-expressed with a Gαi-coupled short form D2 dopamine receptor (D2s-R) in CHO cells. AC assays were performed in the presence of forskolin (100 μM) at the indicated concentration of a D2s-R-selective agonist (quinpirole, Quin). As shown in Fig. 2B, inhibition of ACVI-ΔA87 by D2s-R stimulation was more significant than that of wild-type ACVI at high concentrations of Quin. This observation indicates that partial truncation of the N-terminal domain might cause ACVI to be more sensitive to Gαi-mediated inhibition. To verify this hypothesis, we next examined the inhibitory effect of myristoylated Gαi on the recombinant Gαi protein on the Gαi-evoked activities of wild-type ACVI and ACVI-ΔA87. Myristoylation of the recombinant Gαi protein produced in the absence of N-myristoyltransferase (Fig. 3A) was verified by its faster mobility in 9% SDS-PAGE gels containing 4 μl urea when compared with those of the unmodified Gαi proteins prepared in the absence of N-myristoyltransferase (Fig. 3A). Consistent with the above observation, inhibition of ACVI-ΔA87 by the myristoylated Gαi was much more significant than that of wild-type ACVI (Fig. 3B). No significant difference in the IC₅₀ values of the activated/myristoylated Gαi for the wild-type ACVI and ACVI-ΔA87 was observed (16.6 and 21.5 nM, respectively). However, the extent of maximal inhibition of ACVI-ΔA87 by the activated/myristoylated Gαi was markedly higher than that of wild-type ACVI (71.5% and 46.4% for ACVI-ΔA87 and the wild-type ACVI, respectively) (Fig. 3B), further supporting a functional role of the N-terminal domain of ACVI in Gαi-evoked inhibition.

We next replaced the N-terminal domain of ACVI by that of type V adenylly cyclase (ACV), the closest isozyme to ACVI in the AC superfamily. Except for the N-terminal domain, the C1α and C2 domains of ACVI and ACV are highly homologous (23). Using a two-step PCR-based mutagenesis method, we replaced the N terminus (aa 1–160) of rat ACVI with the N terminus (aa 1–242) of rat ACV. The resultant mutant (designated ACVI-N5) exhibited a larger size than that of wild-type ACVI because of the longer N terminus it possesses (Fig. 4A). In line with our previous finding (14), which suggested that the N terminus of ACVI does not contribute to the catalytic core complex, major enzymatic properties (i.e. Vₘₐₓ and EC₅₀ values of forskolin and Gαi protein) of ACVI-N5 were very similar to those of wild-type ACVI (Table I). In contrast, when co-expressed with D2s-R in CHO cells, D2s-R-mediated inhibition of ACVI-N5 was much less effective at both low (10 nM) and high (10 μM) doses of a D2s-R agonist (Quin; Fig. 4B). Because replacement of the N terminus domain markedly altered its response to Gαi-mediated inhibition of ACVI, these results strengthened our hypothesis that the N terminus of ACVI plays an important role in Gαi-mediated inhibition.

Heissauer and colleagues (19) previously reported that the Gαi protein interacts with the C1α domain of ACV. It is therefore possible that the N terminus of ACVI might modulate Gαi-mediated inhibition by interacting directly with the C1α domain. We first prepared a C1α domain of ACVI (aa 364–587, designated C1α-1) which comprised the partial Gαi-binding domain based on a previous study of ACV (19) using an in vitro TnT translation system. As shown in Fig. 5A, interaction be-
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Fig. 2. Truncation of the N terminus of ACVI alters its response to a Gs-coupled receptor. A, membrane proteins (100 μg per lane) from CHOP cells expressing the wild-type (WT) or the N terminus-truncated ACVI mutant (ACVI–ΔA87) were subjected to Western blot analysis using AC6D. The thick and thin arrows indicate the wild-type and truncated ACVI proteins, respectively. B, membrane fractions collected from CHOP cells transiently transfected with D2s-R plus ACVI-WT or ACVI–ΔA87 were used for the AC activity assay. AC activity evoked by forskolin (100 μM) was measured in the presence of a D2s-selective agonist (Quin) at the indicated concentrations. Values are expressed as percentages of forskolin-evoked ACVI activity measured in the absence of Quin (574.6 ± 105.7 pmol/min/mg) for wild-type ACVI and 673.6 ± 117.3 pmol/min/mg for ACVI–ΔA87), and represent the mean ± S.E. of four independent experiments.

Differences between the Quin-mediated inhibition of forskolin-evoked AC activity of WT and those of ACVI–ΔA87 at 3 and 10 μM are statistically significant: *, p < 0.05, compared with WT at the indicated concentration (by Student’s t test).

tween C1a-1 and the activated/myristoylated Go, protein, which harbored an H6 tag was demonstrated by an Ni-NTA resin pull-down assay. Thus, the C1a domain of ACVI, similar to ACV, bound to the Go, protein. Moreover, recombinant C1a domains synthesized using this in vitro translation system appeared to exhibit the proper conformation. We next performed the in vitro binding analyses of the recombinant N (aa 1–160, designated N1–160) and the C1a-1 domain. Using an antibody that specifically interacts with the N-terminal domain of ACVI (34), the C1a-1 fragment could be co-immunoprecipitated with N1–160 (Fig. 5B). Note that a smaller degradation protein sometimes appeared in the in vitro-translated C1a-1 preparation (Fig. 5B); we therefore constructed another C1a domain (aa 364–575, designated C1a-2) based on a previous study of AC7 (7). The C1a-2 protein comprises the predicted Go,-interacting domain of ACVI as does the C1a-1 protein and an H6 tag fused to its N terminus. As shown in Fig. 5C, C1a-2 was very stable and could also be co-immunoprecipitated with N1–160 as could C1a-1. The H6 tag did not appear to affect the interaction between the N and C1a domains in that both C1a-1 and C1a-2 proteins could be co-immunoprecipitated by N1–160. The C1a-2 protein therefore was used in the following experiment.

To determine the interaction region on the N terminus with the C1a domain, a truncated N-terminal domain (aa 1–86, designated N1–86) was produced for the in vitro binding assay. As shown in Fig. 5D, N1–86 was also effectively co-immunoprecipitated with the recombinant C1a-2 domain as was the N1–160 fragment. Under the conditions used, ~10% of the C1a-2 protein in the reaction was pulled down with both N1–160 and N1–86. The C1a-interacting region of the N terminus might therefore reside in the region containing aa 1–86.

We further performed experiments to determine where on the C1a domain the N-terminal domain binds. Three shorter recombinant proteins comprising different portions of the C1a-2 protein (aa 364–505, 399–540, and 434–575, designated C1a-3, C1a-4, and C1a-5, respectively) were prepared in vitro. As shown in Fig. 6, the N1–160 protein could immunoprecipitate all three of these recombinant C1a proteins tested. The overlapping region (aa 434–505) of these three recombinant C1a proteins thus appeared to contain the structural determinants for the binding of the N-terminal domain.

Based on the available crystallographic structure of the catalytic complex of ACs (7), we built a computer model of the catalytic core composed of the C1a and C2 domains of ACVI (Fig. 1B). Dessauer and colleagues (19) reported that Go, binds to the C1a domain at the cleft formed by the α2 and α3 helices. Mutations of several residues located in these two helices caused a significant reduction in the affinity between Go, and
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ACV. Our results demonstrated that the N terminus binding region of the C1a domain (aa 434–505) containing the α3 helix, which is located on the surface of the catalytic core complex (Fig. 1B), is likely accessible to the N-terminal domain of ACV. Amino acid alignment analysis with ACV suggested that two ACV-equivalent residues (Leu-472 and Val-476) in the α3 helix of the C1a domain of ACVI might be important for interaction with the Gαi protein (Supplementary Materials, Fig. S1). To test whether Leu-472 and Val-476 on the α3 helix might also be important for the interaction of the N and C1a domains, we created a recombinant C1a-2 mutant carrying mutations at Leu-472 and Val-476 (designated mC1a-2). As observed by pull-down assays, mC1a-2 did not interact with the N terminus as did the recombinant wild-type C1a-2 protein (Fig. 7). Thus, Leu-472 and Val-476 located in the α3 helix of the C1a domain of ACVI might play a critical role in the interaction with the N-terminal domain.

To examine whether Leu-472 and Val-476 were important for interacting with the Gαi protein, we mutated Leu-472 and Val-476 into alanine in the full-length ACVI and the N terminus-truncated ACVI mutant (ACVI-ΔA87). The resultant mutants (designated ACVI-L472A-V476A and ACVI-ΔA87-L472A-V476A, respectively) were then subjected to regulation by myristoylated Gαi. With the N terminus being truncated, which would free the C1a domain from binding to the N terminus, the Leu-472/Val-476 mutation markedly reduced the inhibition evoked by Gαi at all dosages examined (ACVI-ΔA87-L472A-V476A versus ACVI-ΔA87, Fig. 8). With an intact N terminus, the effect of the Leu-472/Val-476 mutation (ACVI-L472A-V476A versus wild type, Fig. 8) depended on Gαi dosages: little or no effect at low dosages (22 and 44 nM, 1- to 2-fold of the IC50 values of ACVI) and moderate reduction at high dosages (175 and 350 nM, 10- to 20-fold of the IC50 values of ACVI). In combination, truncation of the N terminus did not alter the inhibition of low dosage Gαi on a mutant for which a potential Gαi-binding site involving Leu-472 and Val-476 had been mutated, but as the dosage of Gαi increased substantially, increased inhibition became evident (ACVI-ΔA87-L472A-V476A versus ACVI-L472A-V476A, Fig. 8).

**DISCUSSION**

In the present study, we investigated the contribution of the N-terminal domain of ACVI to Gαi-mediated inhibition by determining 1) the effect of Gαi-mediated regulation on various ACVI mutants of a truncated N terminus and/or amino acid mutations of the C1a domain at its N- and Gαi-binding site and 2) the in vitro interaction of the recombinant N terminus and the C1a domain with which the Gαi protein binds. Enzymatic analyses revealed that the absence of aa 1–86 created a conformation of ACVI, which enabled the resultant mutant to be more severely inhibited by a Gαi-coupled receptor (D2s-R; Fig. 2) or the myristoylated recombinant Gαi protein (Fig. 3). Sub-

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**Fig. 3. Involvement of the N terminus of ACVI in the regulation of Gαi-mediated regulation.** A, recombinant human Gαi, proteins (0.1 µg) produced in the absence (left lane) or presence (right lane) of N-myristoyl transferase (28) in bacterial BL21-DE3 were subjected to electrophoresis on a 9% SDS-polyacrylamide gel containing 4 M urea, followed by Western blot analyses using an anti-Gαi antibody as described under “Experimental Procedures.” The thick and thin arrows indicate the lipid-modified and unmodified Gαi, proteins, respectively. B, membrane fractions collected from CHO/P cells transiently transfected with ACVI-WT or ACVI-ΔA87 were used for the AC activity assay. AC activity evoked by the activated Gαi protein (0.19 µM) was measured in the presence of activated/myristoylated recombinant Gαi, protein (Gαi,3) at the indicated concentrations. Values are expressed as percentages of Gαi,3-evoked ACV activity measured in the absence of Gαi,3 (146.2 ± 30.3 pmol/min/mg) for wild-type (WT) ACVI and 108.7 ± 21.2 pmol/min/mg for ACVI-ΔA87 and represent the mean ± S.E. of four independent experiments. Differences between the Gαi,3-mediated inhibition of Gαi,3-evoked AC activities of WT and those of ACVI-ΔA87 are statistically significant: *, p < 0.05, compared with WT at the indicated concentration (by Student’s t test).
The N-truncated ACVI variant (ACVI-DΔA87) evoked by Gαi (Fig. 8) and markedly hampered the binding of the N terminus to the C1a domain (Fig. 7). These two residues thus play an important role in Gαi-mediated inhibition in the absence of an intact N terminus and apparently also act as a modulator of the binding between the N and C1a domains. Mutations of Leu-472 and Val-476 in the full-length ACVI might not cause significant effects on Gαi-mediated regulation, because such mutations could elicit dual effects: inactivation of a Gαi-interacting site and dissociation of the N and C1a domains to enable the inactivated Gαi-interacting site to be more accessible to Gαi. In line with this model, we observed no difference in Gαi-mediated inhibition of the enzyme between mutants ACVI-DΔA87-L472A-V476A and ACVI-L472A-V476A at low dosages (i.e. around the IC50 value) of Gαi proteins (Fig. 8), presumably because truncation of the N terminus mainly exposed the Gαi-interacting site of the C1a domain, which had already been inactivated by the mutations of Leu-472 and Val-476. Together with the observations that, at low dosages of Gαi, the Gαi inhibition of wild type (ACVI) was significantly reduced by the truncation of the N domain (ACVI-DΔA87) but essentially was not changed by the Leu-472/Val-476 mutation (ACVI-L472A-V476A), we concluded that binding of N to C1a contributed to inhibition of ACVI evoked by low levels of Gαi. The N terminus domain also plays a critical role in Gαi-mediated inhibition at high dosages of Gαi, because its removal enhanced the inhibition of the full-length mutant (ACVI-L472A-V476A). The molecular mechanism underlying such an action of the N terminus at high levels of Gαi (i.e. ~10- to 20-fold of the IC50 value) is currently unclear. Because interaction of N and C1a domains was not observed with the mutations at Leu-472/Val-476 as assessed by the pull-down assay (Fig. 7), N and C1a domains in ACVI-L472A-V476A were likely to be dissociated. In the absence of the binding of N terminus to C1a, truncation of the N terminus retained the ability to enhance Gαi-mediated inhibition at high dosages of Gαi, suggesting that binding to C1a might not be relevant to how the N terminus suppressed the inhibition of ACVI by high dosages of Gαi. It is possible that the N terminus might interact with an additional Gαi-interacting site other than that on the C1a domain. Alternatively, the pull-down immunoprecipitation assays utilized in the present study might not be sensitive enough to detect weak interactions. We cannot rule out the possibility that a weak interaction between N and C1a domains persisted in ACVI-L472A-V476A and moderately hindered the accessibility of the Gαi-interacting site of the C1a domain at high dosages of Gαi. Collectively, data presented herein suggest a novel function of the N terminus of ACVI in Gαi-mediated regulation. At least part of the action of the N terminus was mediated by binding to the α3 helix of the C1a domain, which subsequently might hinder the accessibility of the cleft formed by the α2 and α3 helices of the C1a domain (Fig. 1B) to Gαi at low levels of Gαi protein.

**Table I**

| Enzymatic properties of ACVI mutants |
|-------------------------------------|
|                                    |
|                                      |
| ACVI  |  |  |  |
| Gαi  | EC50 |  |  |
| kmol | M pmol  |
| WT   | 35.3 ± 9.6 | 15.0 ± 2.0 | 72.5 ± 15.4 |
| N5   | 51.5 ± 5.8 | 20.0 ± 4.8 | 142.2 ± 37.4 |

**Fig. 4.** Altering the extent of inhibition mediated by a Gαi-coupled receptor through replacing the N terminus of ACVI with that of ACV. A, membrane proteins (100 μg per lane) from CHOP cells expressing the wild-type (WT) or the ACVI mutant containing the N terminus of ACV (ACVI-N5) were subjected to Western blot analysis using AC6D. The thick and thin arrows indicate wild-type and ACVI-N5 proteins, respectively. B, membrane fractions collected from CHOP cells transiently transfected with D2s-R plus ACVI WT or ACVI-N5 were used for the AC activity assay. AC activity evoked by forskolin (100 μM) was measured in the presence of a D2s-selective agonist (Quin) at the indicated concentrations. Values are expressed as percentages of forskolin-evoked ACV activity measured in the absence of Quin and represent the mean ± S.E. of three independent experiments. Specific comparisons between the Quin-treated and the control group of each ACVI variant were performed using the Dunnett method. b, p < 0.5. Specific comparisons between the WT and the ACVI-N5 mutants under the indicated conditions were performed using the Dunnett method.

**Resolving the full-length N terminus domain of ACVI with that of ACV also markedly altered the inhibition evoked by D2s-R without affecting other enzymatic properties of ACVI (Fig. 4 and Table I). In vitro binding demonstrated that the N domain binds its catalytic core complex at the C1a domain. In addition, the interacting regions located on the N and C1a domains comprised aa 1–86 and 434–505, respectively (Figs. 5D and 6). Computer modeling suggested that this N terminus-interacting region on the C1a domain contains part of the potential Gαi-interacting region (aa 452–478). It is important to note that the C1a-interacting region (aa 1–86) of the N terminus delineated by the in vitro binding assay is consistent with the observation that the N-truncated mutant (ACVI-DΔA87) was more sensitive to Gαi-mediated inhibition, presumably due to the increased accessibility of the Gαi-interacting site on the C1a domain. More interestingly, simultaneous mutation of two residues (Leu-472 and Val-476) reduced the inhibition of the N-truncated ACVI variant (ACVI-DΔA87) evoked by Gαi (Fig. 8) and markedly hampered the binding of the N terminus to the C1a domain (Fig. 7). These two residues thus play an important role in Gαi-mediated inhibition in the absence of an intact N terminus and apparently also act as a modulator of the binding between the N and C1a domains. Mutations of Leu-472 and Val-476 in the full-length ACVI might not cause significant effects on Gαi-mediated regulation, because such mutations could elicit dual effects: inactivation of a Gαi-interacting site and dissociation of the N and C1a domains to enable the inactivated Gαi-interacting site to be more accessible to Gαi. In line with this model, we observed no difference in Gαi-mediated inhibition of the enzyme between mutants ACVI-DΔA87-L472A-V476A and ACVI-L472A-V476A at low dosages (i.e. around the IC50 value) of Gαi proteins (Fig. 8), presumably because truncation of the N terminus mainly exposed the Gαi-interacting site of the C1a domain, which had already been inactivated by the mutations of Leu-472 and Val-476. Together with the observations that, at low dosages of Gαi, the Gαi inhibition of wild type (ACVI) was significantly reduced by the truncation of the N domain (ACVI-DΔA87) but essentially was not changed by the Leu-472/Val-476 mutation (ACVI-L472A-V476A), we concluded that binding of N to C1a contributed to inhibition of ACVI evoked by low levels of Gαi. The N terminus domain also plays a critical role in Gαi-mediated inhibition at high dosages of Gαi, because its removal enhanced the inhibition of the full-length mutant (ACVI-L472A-V476A). The molecular mechanism underlying such an action of the N terminus at high levels of Gαi (i.e. ~10- to 20-fold of the IC50 value) is currently unclear. Because interaction of N and C1a domains was not observed with the mutations at Leu-472/Val-476 as assessed by the pull-down assay (Fig. 7), N and C1a domains in ACVI-L472A-V476A were likely to be dissociated. In the absence of the binding of N terminus to C1a, truncation of the N terminus retained the ability to enhance Gαi-mediated inhibition at high dosages of Gαi, suggesting that binding to C1a might not be relevant to how the N terminus suppressed the inhibition of ACVI by high dosages of Gαi. It is possible that the N terminus might interact with an additional Gαi-interacting site other than that on the C1a domain. Alternatively, the pull-down immunoprecipitation assays utilized in the present study might not be sensitive enough to detect weak interactions. We cannot rule out the possibility that a weak interaction between N and C1a domains persisted in ACVI-L472A-V476A and moderately hindered the accessibility of the Gαi-interacting site of the C1a domain at high dosages of Gαi. Collectively, data presented herein suggest a novel function of the N terminus of ACVI in Gαi-mediated regulation. At least part of the action of the N terminus was mediated by binding to the α3 helix of the C1a domain, which subsequently might hinder the accessibility of the cleft formed by the α2 and α3 helices of the C1a domain (Fig. 1B) to Gαi at low levels of Gαi protein.
The N-terminal domains of AC isoforms are highly variable and are generally considered regulatory domains. For example, the N-terminal domain of AC VIII has been implicated in calmodulin binding and the Ca$^{2+}$-dependent activation of AC VIII (15). We previously showed that the N terminus of ACVI is crucial for PKC-mediated inhibition (14, 16). Removal of the most-N-terminal portion (aa 1–86) of ACVI did not affect its general enzymatic properties (14). In contrast, as demonstrated in the present study, truncation of this region markedly enhanced the regulation mediated by G$\alpha_i$ (Figs. 2 and 3). The N-terminal domain of ACV did not exert a significant effect on the enzymatic properties or on certain regulatory modes (such as the inhibition mediated by G$\alpha_i$ or calcium (12, 35)). Replacement of the N terminus of ACVI with that of ACV did not affect the enzymatic properties of ACVI either (Table I) but markedly altered the inhibition mediated by D2s-R (Fig. 4). This finding further strengthens our hypothesis that, in addition to its involvement in PKC-mediated inhibition, the N terminus of ACVI might also play a neutralizing role in G$\alpha_i$-mediated inhibition. Note that replacement of the N terminus of ACVI with an irrelevant domain bearing no sequence homology can only be expected to alter its function (i.e. G$\alpha_i$-mediated regulation) but would not necessarily create the same enhancing effect as that observed in the N-truncated ACVI mutant (ACVIΔ87, Fig. 2). One possible explanation for such a finding is that the N terminus of ACV is much longer than that of ACVI (242 vs. 160 aa) and thus might impose a steric hindrance over the G$\alpha_i$-interacting site on the C1a domain and reduce the inhibition evoked by D2s-R.

Ample evidence has previously been shown that different G$\alpha_i$-coupled GPCRs exhibit distinct abilities to activate G$\alpha_i$ proteins and thus lead to different extents of G$\alpha_i$-dependent inhibition of AC (36–38). In response to stimulation by different GPCRs, G$\alpha_i$ protein might inhibit only certain, but not all, G$\alpha_i$-sensitive AC isoforms. For example, the D3 dopamine receptor significantly inhibits the activity of ACV but not that of ACVI, which belongs to the same subfamily as ACV. On the contrary, the extents of D2-R-mediated inhibition are similar for ACV and ACVI (39). The mechanism underlying such specificity is not fully understood. One possible explanation suggested by those authors is that the actions of G$\alpha_i$ protein on ACV and ACVI may differ. Alternatively, stimulation of differ-

**FIG. 5.** In vitro characterization of the interaction between the C1a domain and the N terminus of ACVI. A, binding between the recombinant C1a-1 protein and the myristoylated G$\alpha_i$, protein was performed by mixing the [35S]C1a-1 protein (aa 364–587, 12 fmol; synthesized using an in vitro translation system in the presence of [35S]methionine) with 10 μg of myristoylated H6-G$\alpha_i$ bound on Ni-NTA His-Bind® resins or the same amount of the Ni-NTA His-Bind® resins alone as the control. The mixtures were incubated for 2 h at 4 °C to allow complex formation. After extensive washes, the protein complexes were separated on 12.5% SDS-PAGE and analyzed by Western blot analysis to determine the levels of G$\alpha_i$, (upper panel) and by autoradiography to visualize [35S]C1a-1 (lower panel). The thick and thin arrows indicate the G$\alpha_i$, protein and C1a-1 protein, respectively. B, the N-terminal domain of ACVI (aa 1–160, N1–160) and the C1a-1 domain were produced using an in vitro translation system (TNT) in the presence of [35S]methionine. Production of these proteins was visualized by loading 1 μl of each TNT reaction mixture into the indicated lane. For in vitro binding analysis, 12 fmol of the N1–160, 8 fmol of the C1a-1 proteins were incubated for 60 min at 30 °C to allow complex formation. Immunoprecipitation was performed using an antiserum against the N terminus of ACVI (AC6N (34)). The protein complexes were separated by 15% SDS-PAGE, dried, and visualized by autoradiography. The thick and thin arrows indicate C1a-1 and N1–160, respectively. The star indicates a degradation product of C1a-1. C, in vitro binding analyses of the N1–160 and C1a-2 (aa 364–587, with an H6 tag fused to its N terminus domain) were performed and analyzed as in B, except that the protein complexes were separated by 12.5% SDS-PAGE. The thick and thin arrows indicate C1a-2 and N1–160, respectively. D, in vitro binding analyses of N1–86 and C1a-2 were carried out and quantified as in B. The thick and thin arrows, respectively, indicate C1a-2 and N1–86. Ni-NTA, Ni-NTA His-Bind® resin.
Amino acids 434–505 comprising the N terminus-interacting domain. Recombinant N terminus (aa 1–160, N1-160) and three C1a fragments (aa 364–505, 399–540, and 434–575 for C1a-3, C1a-4, and C1a-5, respectively) were produced using the in vitro TNT system in the presence of [35S]methionine. The arrowhead therefore is smaller than C1a-2. The does not contain an H6 tag fused to its N terminus. The asterisk indicates the N terminus of AC isozymes. For the fmol of the N 1

Recombinant N terminus (aa 1–160 protein and 12 fmol of the indicated C1a-1 variant were incubated for 60 min at 30 °C to allow complex formation. Immunoprecipitation was performed using an antiserum against the N terminus of ACVI (AC6N (34)). The protein complexes were separated by 15% SDS-PAGE and visualized by autoradiography. The thick and thin arrows indicate the C1a-1 variant and N 1

Fig. 6. Effect of mutations of Leu-472 and Val-476 on the Gαi-mediated inhibition of wild-type ACVI and the N-truncated ACVI variant. Membrane fractions collected from CHOP cells transiently transfected with the indicated ACVI variant were used for the AC activity assay. AC activity evoked by the activated recombinant Gαi protein (95 nM) was measured in the absence or presence of the activated/myristoylated recombinant Gαi1 protein (Gαi1) at the indicated concentrations. Values are expressed as percentages of Gαi-evoked ACVI activity measured in the absence of Gαi1 (283.5 ± 48.9 pmol/(min·mg) for wild-type (WT) ACVI; 221.0 ± 83.0 pmol/(min·mg) for ACVI-∆A87; 333.6 ± 61.8 pmol/(min·mg) for ACVI-L472A-V476A; and 268.5 ± 102.2 pmol/(min·mg) for ACVI-∆A87-L472A-V476A) and represent the mean ± S.E. of 4–6 independent experiments. Statistical significance was evaluated by one-way analysis of variance followed by the Student-Newman-Keuls method. a, p < 0.05. Specific comparisons between variants carrying the intact and the truncated N terminus (i.e. ACVI wild-type versus ACVI-∆A87, or ACVI-L472A-V476A versus ACVI-∆A87-L472A-V476A) are shown. b, p < 0.05. Specific comparisons between variants with and without mutations at Leu-472/Val-476 (i.e. ACVI wild-type versus ACVI-L472A-V476A, or ACVI-∆A87 versus ACVI-∆A87-L472A-V476A) are shown.

Fig. 7. Mutation of the potential Gαi-interacting residues (Leu-472 and Val-476) in the α3 helix hampered the binding of the C1a domain to the N terminus. The recombinant N1-160 protein and the indicated C1a-2 fragments were produced using the in vitro TNT system in the presence of [35S]methionine. In vitro binding analysis was carried out as described in Fig. 5. Note that, unlike C1a-2, mC1a-2 does not contain an H6 tag fused to its N terminus domain, and therefore is smaller than C1a-2. The thick and thin arrows indicate C1a-2 and mC1a-2, respectively. The arrowhead indicates the N terminus. The asterisk indicates a minor degradation product of mC1a-2.

Current GPCRs might cause specific conformational changes in Gαi, and, subsequently, lead to selective regulation of AC isoforms. As discussed above, although the transmembrane regions and catalytic core complexes of AC isoforms are similar, they diverge in their N-terminal domains. This raises the possibility that the N terminus of AC isoforms might influence their response to Gαi by modulating the Gαi-binding site on the catalytic core complex. The association of its α3 helix and the C1/C2 catalytic complex endows ACVI with a fine-tuned, regulatory mode for Gαi isoforms, which would contribute to the lower effectiveness of N terminus truncation of ACVI as seen in Fig. 2B.

The molecular basis underlying the regulation of ACVI by Gαi has heretofore not been extensively investigated. Based on a mutational analysis of ACV (19), the basic Gαi-interacting region of ACV might reside in the cleft formed by the α2 and α3 helices of the C1a domain. Our mutation analyses and computer modeling revealed that the N terminus might interact with C1a at the α3 helix. In addition, mutations of Leu-472 and Val-476 located in the α3 helix caused dual effects, including a reduction in Gαi-mediated inhibition in the absence of an intact N terminus (Fig. 8) and dissociation of the N and C1a domains (Fig. 7). These two residues (Leu-472 and Val-476) thus appeared to be important for the interaction between the C1a and N domains, and between the C1a domain and Gαi. Nevertheless, the exact binding sites on the C1a domain for the Gαi-mediated inhibition between wild-type ACVI and the N terminus-truncated ACVI variant (ACVI-∆A87) could not be reversed by increasing the concentration of the D2 agonist (Quin) or the activated/myristoylated Gαi (Figs. 2 and 3). Binding of the N terminus to the C1a domain thus appears to negatively regulate the inhibition evoked by low levels of Gαi in a non-competitive manner. We were unable to further characterize the binding of the N terminus domain and Gαi to the C1a through Gαi2, but not Gαi1 (40, 41). The endogenous Gαi isoform employed by the D2s-R in CHOP cells to mediate the inhibition of ACVI therefore might not be Gαi1. It is plausible that the regulatory mode of ACVI by Gαi might differ slightly from those by other Gαi isoforms, which would contribute to the lower effectiveness of N terminus truncation of ACVI as seen in Fig. 2B.
domain in vitro due to the extremely low expression and instability of the recombinant N-terminus protein (data not shown). Consistently, sequence analyses (PONDR, Molecular Kinetics, Pullman, WA) predicted that the C1a-interacting region (aa 1–86) on the N-terminus is likely to be intrinsically unstructured (42). The potentially highly disordered structure of the N-terminus also suggests that it is likely to form a complex with other peptides (e.g. the C1a domain) to stabilize its structure. It remains to be determined whether the interaction between the N and the C1a domains can be regulated. It is possible that the N-terminus of ACVI might play a fine-tuning role and disassociate from the catalytic core complex under certain regulating condition, which subsequently leads to increased accessibility of the Gαi protein to its interacting cleft on the C1a domain. Exposure of an active site of an enzyme covered by its regulatory domain upon stimulation has many precedent examples, including protein kinase C (PKC). Activation of conventional PKC causes a conformational change that leads to exposure of the kinase domain originally covered by its regulatory domain in the resting stage (31).

In summary, we provide evidence to demonstrate that the N-terminus of ACVI interacts with its catalytic core and plays an important role in the regulation of Gαi-mediated inhibition. These findings further attribute the functional role of the N-terminal domain to the heterogeneity of the AC superfamily and add additional dimensions to the specificity of Gαi-mediated inhibition.

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An Important Functional Role of the N Terminus Domain of Type VI Adenylyl Cyclase in G α4-mediated Inhibition
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