Regulation of Type VI Adenylyl Cyclase by Snapin, a SNAP25-binding Protein*

Jui-ling Chou‡§, Chuen-Lin Huang‡, Hsing-Lin Lai‡, Amos C. Hung‡, Chen-Li Chien‡, Yu-Ya Kao§, and Yijuang Chern‡§

From the ‡Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan, the §Institute of Neuroscience, National Yang-Ming University, Taipei 112, Taiwan, and the ¶Department of Medical Technology, Yuanpei University of Science and Technology, Hsinchu 300, Taiwan

In the present study, we used the N terminus (amino acids 1–160) of type VI adenylyl cyclase (ACVI) as bait to screen a mouse brain cDNA library and identified Snapin as a novel ACVI-interacting molecule. Snapin is a binding protein of SNAP25, a component of the SNARE complex. Co-immunoprecipitation analyses confirmed the interaction between Snapin and full-length ACVI. Mutational analysis revealed that the interaction domains of ACVI and Snapin were located within amino acids 1–86 of ACVI and 33–51 of Snapin, respectively. Co-localization of ACVI and Snapin was observed in primary hippocampal neurons. Moreover, expression of Snapin specifically eliminated protein kinase C (PKC)-mediated suppression of ACVI, but not that of cAMP-dependent protein kinase (PKA) or calcium. Mutation of the potential PKC and PKA phosphorylation sites of Snapin did not affect the ability of Snapin to reverse the PKC inhibitory effect on ACVI. Phosphorylation of Snapin by PKC or PKA therefore might not be crucial for Snapin action on ACVI. In contrast, SnapinΔ33–51, which harbors an internal deletion of amino acids 33–51 did not affect PKC-mediated inhibition of ACVI, supporting that amino acids 33–51 of Snapin comprises the ACVI-interacting region. Consistently, Snapin exerted no effect on PKC-mediated inhibition of an ACVI mutant (ACVIΔA87), which lacked the Snapin-interacting region (amino acids 1–86). Snapin thus reverses its action via direct interaction with the N terminus of ACVI. Collectively, we demonstrate herein that in addition to its association with the SNARE complex, Snapin also functions as a regulator of an important cAMP synthesis enzyme in the brain.

Adenylyl cyclases (ACs) are a family of enzymes that produce cyclic AMP (cAMP) from ATP upon extracellular stimulation. To date, at least 9 membrane-bound ACs have been isolated and characterized (1). These enzymes are capable of integrating positive and negative signals that act directly through stimulation of G protein-coupled receptors (GPCRs) or indirectly via intracellular signaling molecules in isoyme-specific patterns. In addition, the regulatory properties and expression patterns of different AC isoforms greatly diverge and may account for the distinctive cell- and tissue-specific responsiveness of ACs. Recently, several different proteins, including RGS2 and the protein associated with Myc (PAM), have been shown to interact and modulate activity of different AC isoforms (2, 3), adding additional dimensions to the isoyme-specific regulation of the AC superfamily.

Except for the newly identified soluble AC, all membrane-bound AC members share a primary structure consisting of 12 transmembrane regions and 3 large cytoplasmic domains (N, C1a/b, and C2). The C1a and C2 domains, which form the catalytic core complex, are highly conserved and are homologous to each other. The N-terminal domains of ACs, in contrast, are variable among ACs, and have been demonstrated to play specific regulation of the AC superfamily. To date, at least 9 membrane-bound ACs have been isolated and characterized (1). These enzymes are capable of integrating positive and negative signals that act directly through stimulation of G protein-coupled receptors (GPCRs) or indirectly via intracellular signaling molecules in isoyme-specific patterns. In addition, the regulatory properties and expression patterns of different AC isoforms greatly diverge and may account for the distinctive cell- and tissue-specific responsiveness of ACs. Recently, several different proteins, including RGS2 and the protein associated with Myc (PAM), have been shown to interact and modulate activity of different AC isoforms (2, 3), adding additional dimensions to the isoyme-specific regulation of the AC superfamily.

Published, JBC Papers in Press, August 19, 2004, DOI 10.1074/jbc.M407206200

Received for publication, June 28, 2004

* This work was supported by Grants NSC92-2320-B001-011 and NSC90–2320-B001-009 from the National Science Council and Grants NHRI-EX92-9203NI and NHRI-EX93-9203NI from the National Health Research Institutes and Academia Sinica, Taipei, Taiwan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains Supplementary Materials.

‡ To whom correspondence should be addressed. Tel.: 886-2-26523913; Fax: 886-2-27829143; E-mail: bmychern@ibms.sinica.edu.tw.

¶ The abbreviations used are: AC, adenylyl cyclase; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; SNARE, soluble NSF attachment protein receptors; BSA, bovine serum albumin; MBS, maleimido-benzoyl-N-hydroxysuccinimide ester; ACVI, type VI adenylyl cyclase; DIV, days in vitro; MBP, maltose-binding protein.
PKC loses its ability to suppress ACVI activity in the presence of Snapin. These findings demonstrate, for the first time, a fine-tuning mechanism for the cAMP production system by the vesicle-transporting machinery in the brain.

**EXPERIMENTAL PROCEDURES**

**Materials**— Forskolin, cAMP, ATP, progaglandin E1, thapsigargin, and essential amino acids were obtained from Sigma. Purified PKA, thapsigargin, and PKC were purchased from Calbiochem (La Jolla, CA). CellTrackerTM CM-DX was obtained from Molecular Probes (Eugene, OR). MgCl2 and other chemicals were obtained from Merck (Darmstadt, Germany) unless stated otherwise.

**Yeast Two-hybrid Experiments and Library Screening**—The cDNA of rat ACVI (9) was kindly provided by Dr. R. Iyengar (Department of Pharmacology, Mount Sinai School of Medicine). The DNA fragment encoding the N-terminal domain of ACVI (amino acids 1–160, N1–160) was amplified by the polymerase chain reaction (PCR; see Supplemental Materials, Table S-1), subcloned into the pcAS2-1 vector, and served as the bait to screen a mouse brain cDNA library using the Matchmaker Two-hybrid System 2 (Clontech, Palo Alto, CA) following the manufacturer’s instructions. The identified cDNA clones were analyzed by nucleotide sequencing and functional annotation using standard bioinformatics algorithms.

**Plasmid Construction**—A 0.75-kb BglII fragment of pACT2-Snapin, which contained the entire coding region of Snapin was ligated into the EcoRI site of a pcDNA3 vector (Clontech) resulting in pcDNA3-Snapin. The DNA fragment encoding the N-terminal domain of ACVI (amino acids 1–160, N1–160) was amplified by PCR using the primers listed under “Supplemental Materials” (Table S-1), and subcloned either into the NotI and BamHI sites of pET-11d (Novagen, Madison, WI), pET-11d-N1–160, pET-11d-N1–160, pET-11d-N1–160, pET-11d-Snapin1–95, pET-11d-Snapin1–95, or pET-11d-Snapin1–95, into the EcoRI and XhoI sites of pMAL-C2X (pMAL-Snapin; 14) using standard molecular biology methods, or into the pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) using a TA expression kit (Invitrogen; pcDNA3.1-Snapin1–95, pcDNA3.1-Snapin1–95).

The Snapin-Delta-51 mutant, Snapin-S50A, and Snapin-T-30A were constructed by site-directed mutagenesis of pcDNA3-Snapin. (Ref. 5; Table S-1) with pcDNA3-Snapin as the DNA template. The identified cDNA clones were analyzed by nucleotide sequencing and functional annotation using standard bioinformatics algorithms.

**Production and Biotinylation of the Polyclonal Anti-Snapin Antibody**—The oligopeptide (S118–136) CARRRLMDGSGVYPPGSPSK, corresponding to amino acids 118–136 of mouse Snapin was purchased from Genosys (The Woodlands, TX) and conjugated to bovine serum albumin (BSA) using the sulfo-ethylmaleimide ester (MBS) as described by Harlow et al. (19). The antiserum was generated by injecting male New Zealand White rabbits with the BSA-conjugated peptide using standard procedures (19). The resultant antibody was designated SNA-c. To remove the potentially existing anti-BSA IgG, SNA-c antiserum was preabsorbed with 1% BSA in phosphate-buffered saline at 4 °C overnight. To characterize the specificity of the SNA-c antibody, the antibody was conjugated to an irrelevant protein, ovalbumin, using MBS as described above and used for competition experiments. The addition of an excess amount of the peptide antigen (S118–136) conjugated to an irrelevant protein (ovalbumin; 1 mg/ml) completely abolished the immunoreactivity of Snapin in the brain and caused the disappearance of an immunoreactive band of 15 kDa, the predicted molecular mass of Snapin, in the plasma membrane fraction of Sf21 cells (Fig. S-1). For double immunohistochemical staining, the SNA-c antibody was biotinylated. Briefly, the SNA-c antibody was first purified by a protein A-Sephadex column, and then incubated with NHS-LC-Biotin (3 mg/ml; Pierce) at a 1:15 molar ratio. Immunohistochemical staining of the rat brain using the biotinylated SNA-c antibody exhibited an identical expression profile of Snapin as that by the original SNA-c antiserum in rat brains (data not shown). The manufacturer’s instructions were followed for the manufacture of the SNA-c antibody.

**Immunoprecipitation and Western Blotting**—Expressions of wild-type and mutant ACVI were carried out in a recombinant baculovirus-driven SF21 cell system following the manufacturer’s protocol (BD Pharmingen, San Diego, CA) as described elsewhere (8). ACVI proteins were purified by immunoprecipitation with an anti-ACVI antibody (SNA-c) described above. Briefly, 0.3 mg of total protein (SSS-labeled proteins was harvested by incubating the fixed cells in EN’HANCE (NEN TM Life Science Products, Zaventem, Belgium) for 30 min. The complex was first bound to amylase resin (30 μl) and then incubated with the TNT lyases containing [35S]methionine-labeled recombinant proteins (25–60 fmol) at 30 °C for 1 h, followed by addition of 30 μl of the AC6N antibody (10-μg) to Snapin (10 μg) and incubation at 4 °C for 2 h with gentle agitation. The immunocomplexes were purified, extensively washed with 1% SDS-PAGE gels, and then resolved by SDS-PAGE (15%). The bands were visualized using autoradiography.

**Immunohistochemistry**—Double immunostaining of ACVI and Snapin was conducted as detailed elsewhere (20) with modifications. In a 150 mm dish, cells as described above were seeded with a primary anti-ACVI antibody (ACSN, 1:1000; 10) followed by incubation with a goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (1:400), blocked using the avidin D blocking solution (6 μg/ml, avidin/biotin blocking kit, Vector Laboratories, Burlingame, CA) for 30 min and the biotin blocking solution (6 μg/ml, avidin/biotin blocking kit) for 30 min, followed by incubation with the biotinylated SNA-c antibody (1:150), followed by staining with streptavidin conjugated with Alexa Fluor® 568 (1:1000).
Interaction of Type VI Adenylyl Cyclase with Snapin

**A**

- Yeast two-hybrid interaction assay: Y187 yeast transformed with the indicated plasmids in selection plates (-Trp, -Leu) is shown. The black color represents the expression of the reporter (β-galactosidase). Co-transformation of pVA3-1 and pDT1-1 provided by the manufacturer was included as a positive control. Note that the co-transformation of neither Snapin and pVA3-1, nor Snapin and pDT1-1, nor N1-160 and an empty vector (pACT2) caused the expression of β-galactosidase.

**B**

- Co-immunoprecipitation of N1-160 with Snapin. Recombinant N1-160, Snapin, and uORF5 (an irrelevant protein) were produced using the in vitro Tnt system in the presence of [35S]methionine. Production of these proteins was visualized by loading 1 μl of each Tnt reaction mixture (0.001–0.002 pmol) into each lane as shown on the 3 right-most lanes. For co-immunoprecipitation, recombinant N1-160 (0.007 pmol) and [35S]Snapin (0.005 pmol) were incubated for 60 min at 30 °C to allow complex formation. Immunoprecipitation was performed using anti-N1-160 antiserum (IP with AC6N, 10) or normal rabbit serum (IP with NRS) as indicated. The bound proteins were separated by 15% SDS-PAGE, dried, and autoradiographed. C, co-immunoprecipitation of full-length ACVI with Snapin. ACVI was produced in SF21 cells and purified immunologically with an anti-ACVI antibody (AC6D, 8) or AC6D pre-absorbed with the antigen (recombinant C2 domain containing amino acids 987–1187 of ACVI; designated CON). For co-immunoprecipitation, purified ACVI and [35S]Snapin (0.005 pmol) were incubated for 60 min at 30 °C to allow complex formation and were analyzed by Western blot analysis to determine the levels of ACVI (top panel), and by autoradiography to visualize [35S]Snapin (bottom panel). Note that the 2 ACVI-immunoreactive bands in the top panel respectively represent glycosylated and unglycosylated ACVI proteins in SF21 cells (41).

**C**

For triple immunostaining with ACVI, Snapin, and MAP2 in primary hippocampal neurons, cells were blocked with 3% bovine serum albumin for 1 h, stained with a monoclonal anti-MAP2 antibody (1:1000, Chemicon, Temecula CA) at 4 °C overnight, and then incubated in a goat anti-mouse IgG conjugated to Cy5 (1:200, Jackson ImmunoResearch, West Grove, PA) for 2 h. After extensive rinsing, staining of ACVI, and Snapin was carried out as described above. For the triple immunostaining with ACVI, Snapin, and DiI, staining with ACVI and Snapin was carried out as described above. Cells were then stained with DiI (10 ng/μl) in phosphate-buffered saline for 1 h at room temperature, followed by extensive washing with phosphate-buffered saline to remove excess DiI. Patterns of double or triple immunostaining were analyzed with the aid of a laser confocal microscope (Bio-Rad, MRC-1000). Controls for the specificity of immunofluorescence were determined by omission of the primary antibody from the staining process.

**AC Assay and CAMP Measurement**—AC activity was assayed as previously described (21). The enzyme activity was linear for up to 30 min with up to 40 μg of membrane protein. The ACVI activity was determined as the difference between cyclase activities measured in the membrane fractions collected from CHOP cells transfected with the indicated ACVI expression construct and those from cells transfected with the empty vector alone. For CHOP cells expressing wild-type ACVI, endogenous cyclase activities represented ~40% of the total activity. The absolute values of ACVI activity observed in different transient transfection experiments might have slightly varied due to the number of passages and condition of cells. Nevertheless, the overall pattern of AC regulation was consistent among experiments.

Intracellular CAMP content was determined as described before (21, 22) with slight modification. Cells were washed twice with the Ca2+-free Locke’s solution (150 mM NaCl, 5.6 mM KCl, 5 mM glucose, 1 mM MgCl2, and 10 mM HEPES adjusted to pH 7.4) containing 0.5 mM IBMX and resuspended in the same solution at 1 × 105 cells/0.45 ml of each TNT reaction mixture (0.001–0.002 pmol) into each lane as shown on the 3 right-most lanes. For co-immunoprecipitation, recombinant N1-160 (0.007 pmol) and [35S]Snapin (0.005 pmol) were incubated for 60 min at 30 °C to allow complex formation and were analyzed by Western blot analysis to determine the levels of ACVI (top panel), and by autoradiography to visualize [35S]Snapin (bottom panel). Note that the 2 ACVI-immunoreactive bands in the top panel respectively represent glycosylated and unglycosylated ACVI proteins in SF21 cells (41).
FIG. 2. Identification of the Snapin-interacting site of the N terminus of ACVI and the N1-160-interacting site of Snapin. A, recombinant N1-160, N1-123, and Snapin were produced using the in vitro TNT system in the presence of [35S]methionine. Production of these proteins was visualized by loading 1 μl of the indicated TNT reaction mixture (0.0007–0.002 pmol) into each lane as shown in the four right-most lanes. For co-immunoprecipitation, Snapin (0.003 pmol) with either recombinant N1-160, N1-123, or N1-86 (0.006 pmol, respectively) were incubated for 60 min at 30 °C to allow complex formation. Immunoprecipitation was performed using anti-N1-160 antiserum (AC6N). The bound proteins were separated by 15% SDS-PAGE, dried, and autoradiographed. B, summary of the in vitro binding results of C. C, recombinant N1-160 and various Snapin variants were prepared using the in vitro TNT system in the presence of [35S]methionine. Production of these proteins was visualized by loading 1 μl of the indicated TNT reaction mixture (0.0005–0.0017 pmol) into each lane as shown in the six right-most lanes. For co-immunoprecipitation, recombinant N1-160 (0.006 pmol) and the indicated Snapin variant (0.002–0.004 pmol) were incubated for 60 min at 30 °C to allow complex formation. Immunoprecipitation was performed using anti-N1-160 antiserum (AC6N). The bound proteins were separated by 15% SDS-PAGE, dried, and autoradiographed. D, in vitro binding between N1-160 and Snapin was performed as in C. Addition of a peptide comprising amino acids 33–51 of Snapin (S33–51; 400 μM), but not the peptide containing amino acids 118–136 (S118–136; 400 μM), significantly suppressed binding between Snapin and N1-160. E, level of Snapin binding was first normalized with that of the ACVI in the immunocomplexes (i.e. integrated absorbance units of bound Snapin/ACVI). Values for Snapin binding are expressed as percentages of Snapin binding in the absence of any peptide. Data were generated by quantitative computed densitometry of autoradiograms from three independent experiments using the image analysis software package, ImageQuant v.3.15 (Molecular Dynamics). Values represent the mean ± S.E. Statistical significance was evaluated by one-way analysis of variance. Specific comparisons between the indicated treatment and the control group was performed using the Dunnett method. a, p < 0.05.
#### RESULTS AND DISCUSSION

**Snapin Interaction with the N-terminal Domain of ACVI**—Although the N terminus is variable among different AC members, it is highly conserved between species. The N terminus therefore might mediate developmentally conserved regulatory modes of ACVI. Compared with other members in the AC superfamily, the N terminus of ACVI with 160 amino acids is relatively large and might mediate its functions through interacting with other proteins. To search for proteins that interact with the N-terminal domain of rat ACVI (designated N1–160) in the brain, we used N1–160 as the bait to screen a mouse brain cDNA library using the yeast two-hybrid system. This library was chosen because of its availability and because the N termini of rat and mouse ACVI are highly homologous (96% identify in amino acids). Out of $2 \times 10^6$ clones, 2 independent clones encoding a full-length and a partial fragment (amino acids 33–136, designated SNP33–136) of Snapin were identified. Note that the amino acid sequences of mouse and rat Snapin are identical. Such an interaction was verified by co-transforming the identified pACT2-Snapin construct (full-length or the truncated Snapin33–136) with the original N1–160 bait into yeast Y187 cells. As shown in Fig. 1A, co-transformation of the Snapin variant and N1–160 led to expression of the reporter gene ($\beta$-galactosidase), confirming the interaction between

---

**FIG. 3. Colocalization of ACVI and Snapin in vivo.** A–C, double immunostaining of ACVI and Snapin in 8 DIV hippocampal neurons in a resting condition (basal). D–N, triple immunostaining of Snapin, ACVI, and MAP2 (D–I) or Dil (a membrane-partitioning fluorescent dye, 10 ng/μl; J–N) in eight DIV hippocampal neurons in the presence of external high K$^+$ (56 mM, 5 min). MAP2 is a somatodendritic marker. Colocalization of ACVI and Snapin was evident in somata, dendrites, and growth cone tips. ACVI was visualized by the fluorescein isothiocyanate secondary antibody (green; A, D, J). Snapin was visualized by streptavidin Alexa Fluor® 568 (red, B, E) or streptavidin Alexa Fluor® 647 (blue, K). MAP2 was detected by the Cy5 secondary antibody (blue, G). C, merged images of A and B. F, merged images of D and E. H, merged images of E and G. I, merged images of D, E, and G. M, merged images of J, K, and L. The insets show high magnifications of the indicated regions. Scale bars are 10 μm.
Snaphin did not affect the activities of ACVI

CHOP cells were transiently transfected with an expression construct of ACVI plus an empty control vector (pcDNA3) or an expression vector of Snaphin as indicated. Membrane fractions were prepared from transfected cells, and AC activity evoked by activated recombinant Gα protein or forskolin (100 μM) was evaluated. Values represent the mean ± S.E. of 9–12 determinations from 3–4 independent experiments.

| Stimulation | Snaphin | ACVI activity (pmol/mg/min) |
|-------------|---------|-----------------------------|
| Basal       | –       | 33.8 ± 5.7                  |
| Basal       | +       | 43.2 ± 7.9                  |
| Gα (480 nM) | –       | 37.5 ± 5.0                  |
| Gα (480 nM) | +       | 45.0 ± 5.2                  |
| Forskolin (100 μM) | – | 214.3 ± 54.0               |
| Forskolin (100 μM) | + | 187.9 ± 29.7               |

**Table 1**

Snaphin and N1–160 in yeast. *In vitro* binding of the recombinant N1–160 and Snaphin was demonstrated by co-immunoprecipitation using an anti-ACVI antibody (AC6N). As expected, Snaphin, but not an unrelated UORF5 protein, was co-immunoprecipitated with N1–160 (Fig. 1B). This interaction between N1–160 and Snaphin was specific because in the absence of N1–160, no Snaphin was detected in the immunoprecipitated complex (Fig. 1B). Moreover, full-length ACVI prepared from SF21 cells also bound Snaphin (Fig. 1C). Collectively, these data demonstrate that ACVI interacts with Snaphin through direct interaction at its N-terminal domain.

To define the Snaphin-interacting site of N1–160, we performed *in vitro* binding analyses of the recombinant Snaphin and various lengths of the N-terminal domain of ACVI (Fig. 2A). Recombinant proteins were produced using the *in vitro* TnT system in the presence of [35S]methionine. Complex formation was identified by immunoprecipitation using antiseraum (AC6N) against N1–160. As demonstrated in Fig. 2A, the fragment comprising amino acids 1–86 (N1–86) was effectively co-immunoprecipitated with the recombinant Snaphin as were the full-length N terminus (N1–160) and N1–123. The Snaphin-interacting region of N1–160, therefore might reside in the region containing amino acids 1–86.

We next performed experiments to determine what portion on Snaphin N1–160 was bound using the *in vitro* binding assay as described above. Five recombinant proteins comprising different portions of mouse Snaphin (amino acids 26–136, 33–136, 51–136, 1–95, and 1–115, designated SNF26–136, SNF33–136, SNF51–136, SNF1–95, and SNF1–115, respectively, Fig. 2B) were prepared *in vitro*. As shown in Fig. 2, B and C, N1–160 immunoprecipitated all of the Snaphin variants examined, except for SNF51–136. Since SNF51–136 retained the ability to interact with N1–160, amino acids 33–51 of Snaphin appeared to be crucial for the interaction with N1–160. Furthermore, addition of a synthetic peptide comprising amino acids 33–51 of Snaphin (designated S33–51) abated the interaction between Snaphin and N1–160 (Fig. 2, D and E), confirming that the interactive domain of Snaphin with N1–160 resides in the region containing amino acids 33–51 of Snaphin. The peptide containing amino acids 118–136 of Snaphin (designated S118–136) did not exert a significant effect on the binding of Snaphin to N1–160 and thus served as a control peptide in this experiment (Fig. 2, D and E). Collectively, the ACVI-binding region of Snaphin is located within amino acids 33–51, a region different from that which was used to interact with SNAP25 (12) or SNAP23 (24).

**Colocalization of ACVI and Snaphin**—We previously demonstrated that in the brain, ACVI is expressed in most areas examined and is present in cells with a neuronal phenotype (10). Since the regional expression of Snaphin in the central nervous system (CNS) has not yet been reported, we first set out to examine the expression of Snaphin in the brain. Detailed analysis revealed that similar to the expression of ACVI in the brain, Snaphin was widely expressed in many brain areas with different intensities (Supplemental Materials, Fig. S-1B-G). Double immunohistochemical staining further demonstrated that ACVI was colocalized with Snaphin in the adult rat brain (Supplemental Materials, Fig. S-1H-M), and in primary hippocampal neurons (Fig. 3). Importantly, depolarization of these hippocampal neurons by external high K+ (56 mM, 5 min) did...
not alter the colocalization of ACVI and Snapin (Fig. 3, D–N), which could readily be observed in somas and proximal dendrites. Consistently, in vitro binding of ACVI and Snapin determined by co-immunoprecipitation could be observed both in the absence (Fig. 1C), and presence of calcium (data not shown). Interactions between ACVI and Snapin therefore appeared to be independent of the neuron’s activity. The ACVI and Snapin complexes were also colocalized with MAP2 (Fig. 3I), a somatodendritic marker, indicating their somatodendritic localization. In addition to the plasma membrane of somas (Fig. 3N), colocalization of Snapin and ACVI on the tips of growth cones could also readily be observed (Fig. 3, J–M).

Such an interaction between these two molecules therefore might play an important role in regulating neuronal activity.

Although adenylyl cyclases in general are supposed to exist in plasma membranes, we consistently found that ACVI could also be detected in intracellular compartments (Ref. 10 and this study). Similarly, in cultured hippocampal neurons, immunoreactivities of ACI and ACVIII were found in various subcellular locations, in addition to plasma membranes (25, 26). Transport of ACs therefore might be an important regulatory role for the AC family. This is of particular importance since the ACVI-interacting Snapin is known to associate with the SNARE complex, which plays an important role in intracellular membrane cycling. It is therefore possible that Snapin might mediate the cycling of ACVI through interactions with the SNARE complex.

Many neuromodulators, including those that elevate cAMP contents, have been shown to rapidly modulate synaptic strength. We previously showed that ACVI protein is widely expressed in the brain and may participate in regulation of the classical neurotransmitter systems (10). Interactions between ACVI and Snapin as reported herein further support the potential contribution of ACVI in synaptic transmission. Since the cAMP-dependent pathway has been implicated in neurotransmitter release (27), and because phosphorylation of Snapin by PKA enhances its interaction with SNAP25 and synaptotagmin of the assembled SNARE complex (13), direct interactions between Snapin and a cAMP-synthesizing enzyme (ACVI) might set the stage for enhancement of neurotransmitter release by cAMP. Moreover, a somatodendritic expression pattern of Snapin was found in several brain areas and in primary hippocampal neurons (Supplemental Materials, Fig. S-1C-G; Fig. 3G-I). Thus, Snapin/ACVI complexes might also possess certain postsynaptic functions. For example, SNARE complexes are critical for membrane trafficking (28), and therefore might mediate the incorporation of postsynaptic proteins (such as AMPA receptors) into synapses to facilitate synaptic plasticity (29). More importantly, ample evidence suggests that the cAMP system plays a key role in neuronal plasticity. Specifically, phosphorylation of AMPA receptors by PKA has been shown to regulate the insertion of AMPA receptors into synapses and to contribute to plasticity (30, 31). An association of cAMP pathway components (e.g. AKAP and PKA) with receptors (e.g. AMPA receptors) important for synaptic strength has also been reported at postsynaptic sites (32). Interactions between ACVI and Snapin therefore might provide a previously uncharacterized mechanism to enhance the neuronal plasticity regulated by the cAMP pathway.

During the preparation of this article, several studies that call for the reinvestigation of the role and expression of Snapin were published (24, 33, 34). In contrast to a brain-specific expression pattern (12), Snapin was reported to be a ubiquitously expressed soluble protein, which can be detected in both cytosol and peripheral membrane-associated fractions (24, 34). With additional Snapin-interacting proteins being identified (24, 33, 35, 36), the list of postulated functions of Snapin has...
also been expanded into a broader range of intracellular membrane-fusion events, not just limited to neurotransmitter release as proposed earlier (12, 13). Since ACVI, like Snapin, is also widely expressed in many tissues (9), it will be of great interest in the future to characterize whether the association of ACVI and Snapin might also occur in tissues other than the CNS and whether other functions involving intracellular membrane cycling might be regulated by such a close interaction between the cAMP and SNAREs systems.

**Snapp Selectively Modulates the Inhibition of ACVI by PKC**—Among AC isozymes, ACVI is unique because it exhibits relatively low catalytic activity (37), and most regulatory modes of this enzyme reported to date are negative. Specifically, ACVI can be inhibited by calcium, PKA, PKC, and Gsα protein (1, 7, 8, 23, 24, 38). We first determined whether Snapin exerted a significant effect on the enzymatic properties of ACVI. As shown in Table I, expression of Snapin did not affect the basal, Gsα, or forskolin-evoked activities of ACVI in CHOP cells. We next examined whether interaction with Snapin affected the regulation of ACVI by PKA (7). As shown in Fig. 4A, PKA treatment suppressed the activity of ACVI evoked by Gsα or forskolin in expressed in CHOP cells. Membrane fractions prepared from CHOP cells transfected with the indicated expression constructs were incubated with purified PKCs which contained at least 10 different PKC isoforms including the δ and the ε isoforms that inhibit ACVI (8), and then these were assayed for enzymatic properties. Intriguingly, the presence of wild-type Snapin abolished the PKC-mediated inhibition of ACVI activity (Fig. 5A). Since expression of Snapin did not affect PKC activities (Supplemental Materials, Fig. S-2), Snapin did not exert its action through inhibiting PKC. Moreover, mutation of the only potential PKC phosphorylation site on Snapin (Thr117) into alanine, although it reduced its expression level, did not disable the resultant mutant ability to reverse the inhibitory effect of PKC on ACVI. Since Snapin is a substrate of PKA (13), we next mutated the prominent PKA phosphorylation site (Ser50) of Snapin into alanine. Similarly, the resultant Snapin mutant (Snapin-S50A) was as effective as the wildtype Snapin in reversing PKC-mediated inhibition of ACVI. Phosphorylation of Snapin by PKC or PKA therefore is not crucial for Snapin’s action on ACVI. Conversely, SnapinΔΔA78, which harbors an internal deletion of amino acids 33–51 did not affect PKC-mediated inhibition of ACVI, supporting that amino acids 33–51 of Snapin comprises the ACVI-interacting region as suggested in Fig. 2. Moreover, expression of Snapin did not affect PKC-mediated inhibition of ACVI-ΔA78, an ACVI variant, which lacked the Snapin-interacting region (amino acids 1–86, Fig. 5B and Ref. 5). Direct interaction between Snapin and ACVI therefore is crucial for Snapin action.

To further support the effect of Snapin on PKC-mediated inhibition of ACVI, recombinant Snapin fused to maltose-binding protein (designated MBP-Snapin, Fig. 6A) was prepared to test its ability to reverse the PKC-evoked inhibition of ACVI. As shown in Fig. 6B, recombinant MBP-Snapin, but not MBP alone, effectively pulled down recombinant N1–160 in the in vitro binding assays, suggesting that recombinant MBP-
Snailp likely exhibits the proper conformation for their interaction as that of Snailp. Note that a smaller protein existed in the purified MBP-Snailp preparation (Fig. 6A). This protein appeared to be a degradation product of MBP-Snailp because it was recognized by anti-MBP antiserum but not by anti-Snailp antiserum (SNA-c) which was raised against the most C terminus 18 amino acids of Snailp. Since the interaction between MBP-Snailp and N1–160 could be established (Fig. 6B), the existence of this degradation product was unlikely to interfere with the action of Snailp under the experimental conditions tested. Most importantly, recombinant MBP-Snailp, but not MBP alone, dose-dependently relieved the inhibition of ACVI by PKC (Fig. 6C). This observation further strengthens our hypothesis that Snailp selectively modulates the inhibition of ACVI by PKC.

We previously reported that phosphorylation of ACVI by PKC caused suppression of its activity and subsequently led to reduced cAMP signaling during prolonged stimulation of the A2a adenosine receptor in PC12 cells (8, 21, 39). Further biochemical analyses indicated that the regulatory domain (N1–160) and the catalytic core complex (C1/C2) of ACVI are phosphorylated by PKC, and thus contribute to PKC-mediated inhibition (5, 40). These studies suggest that the 3 cytosolic domains of ACVI might form a regulatory complex (40), and that the N terminus might contribute to its overall conformation. Regulation of ACVI by PKC appears to be sensitive to its conformation since defects in glycosylation at extracellular loops 5 and 6 caused ACVI to be less sensitive to PKC-mediated inhibition (41). Herein, we report that the interaction with Snailp caused ACVI to become insensitive to PKC-mediated suppression (Figs. 5 and 6). It is possible that, by interacting with the N terminus of ACVI, Snailp promotes a conformation of ACVI, which is resistant to PKC-mediated regulation.

Phosphorylation of Snailp itself by either PKC or PKA did not appear to be important for the ability of Snailp to regulate the effect of PKC on ACVI (Fig. 5A). We were unable to determine whether Snailp intervened in the action of PKC by interfering with PKC-mediated phosphorylation of ACVI, because the interaction between membrane-bound ACVI and Snailp in CHOP cells was not strong enough to survive membrane solubilization using detergents, a required step for co-immunoprecipitation of ACVI from transfected CHOP cells (data not shown). Dissociation of assembled protein complexes during detergent solubilization and protein purification is a common problem during preparation of protein complexes (42, 43).

Taken together, these findings demonstrate that the action of Snailp on PKC-mediated regulation of ACVI is selective (Figs. 4–6). It is also of great interest to note that not all, but not all, of the ACVI detected in the brain and primary hippocampal neurons was colocalized with Snapin (Supplemental Materials, Fig. S-1 and Fig. 3). Association with Snapin there-
Regulation of Type VI Adenylyl Cyclase by Snapin, a SNAP25-binding Protein
Jui-ling Chou, Chuen-Lin Huang, Hsing-Lin Lai, Amos C. Hung, Chen-Li Chien, Yu-Ya Kao and Yijuang Chern

J. Biol. Chem. 2004, 279:46271-46279.
doi: 10.1074/jbc.M407206200 originally published online August 19, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407206200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/09/01/M407206200.DC1

This article cites 41 references, 18 of which can be accessed free at
http://www.jbc.org/content/279/44/46271.full.html#ref-list-1