MECHANISMS OF SIGNAL TRANSDUCTION:

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We cloned a cDNA encoding a novel synGAP, synGAP-d (GenBank™ accession number AB016962), from a rat brain cDNA library. The clone consisted of 4801 nucleotides with a coding sequence of 3501 nucleotides, encoded a protein consisting of 1166 amino acids with >99% homology with 1092 amino acid overlaps to synGAP, and contained a 13-nucleotide insertion to the previously reported synGAP mRNAs, which suggested that the clone was a splice variant of synGAP. We also found that there are at least seven variants in the 3′ portion of the synGAP mRNA and that they encoded five different protein isoforms. The coding sequence of these C-terminal variants were classified into α1, α2, β1, β2, β3, β4, and γ, and synGAP-d was classified as the β1 form. The previously reported synGAPs (synGAP-a, -b, and -c and p135synGAP) can be classified as the α1 isoform. All isoforms were expressed specifically in the brain. Unexpectedly, the β isoform, which lacks a C-terminal PSD-95-binding motif ((S/T)XV), was more restricted to the postsynaptic density fraction than the motif-containing α1 isoform. The β isoform did not interact with PSD-95 but specifically interacted with a nonphosphorylated α subunit of Ca^{2+}/calmodulin-dependent protein kinase II through its unique C-terminal tail.

The synaptic Ras-GTPase activating protein synGAP (or p135 synGAP) is a brain-specific protein of about 130 kDa, and the synGAP cDNA was cloned independently by two groups (1, 2). There are three isoforms of synGAP proteins: synGAP-a or p135 synGAP, synGAP-b, and synGAP-c (GenBank™ accession numbers AF058789, AF048976, AF058790, and AF050183, respectively). They are different at the N termini, and p135synGAP has N-terminal and C-terminal variants (GenBank™ accession numbers AF053938 and AF055883, respectively) (1). The synGAP protein is condensed in postsynaptic density (PSD),[1] probably through interaction with the PDZ domains of PSD-95/SAP90 via its C-terminal amino acids. The protein negatively regulates Ras activity by stimulating GTPase activity of Ras. Activity of the synGAP is regulated by the phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII); phosphorylation by CaMKII reduces the synGAP activity, leading to Ras activation by increasing the GTP-bound form of Ras (1). In this respect, synGAP is a key molecule that enables cross-talking between Ca^{2+}/calmodulin and Ras/MAPK signaling pathways. Both signaling pathways are believed to function at the postsynaptic sites *in vivo* (3, 4). A recent study implicated Ras signaling and MAPK in the modulation of synaptic transmission (5–8), and certain neurotrophins, such as brain-derived neurotrophic factor, of which signals are processed by the Ras/MAPK pathway system, are involved in synaptic plasticity (9–11). Thus, similar to the Ras guanine nucleotide-releasing factor (or Ras guanine nucleotide exchange factor) (Ras-GRF or CDC25) (12), synGAP may contribute to synaptic plasticity. Neurofibromin 1 protein, a *Drosophila*-specific RasGAP, is involved in the learning and memory of the fruit fly (13).

In this study we cloned a novel isoform of synGAP, synGAP-d, and further identified various isoforms of synGAP. We also identified the nonphosphorylated form of CaMKII α subunit as a specific binding protein of synGAP-β.

EXPERIMENTAL PROCEDURES

Materials—cDNA λ ZAP II library from rat brain cortex and Bluescript plasmid were purchased from Stratagene (La Jolla, CA); Isogen was purchased from Nippon Gene KK (Tokyo, Japan); DNase I was from Qiagen (Tokyo, Japan); RNA PCR kit (Avian Myeloblastosis Virus) version 2.1 was from Takara Co. Ltd. (Ohtsu, Japan); pGEM-T easy vector was from Promega Corporation (Madison, WI); keyhole limpet hemocyanine, protease inhibitor mixture, and anti-actin were from Sigma; Affi-Gel 10 resin was from Bio-Rad; anti-synGAP (anti-α1) (number 06-900) was from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-CaMKII β subunit antibody was from Life Technologies, Inc.; anti-PSD-95 (P43520) was from Transduction Laboratories (Lexington, KY); anti-neurofilament (70 kDa) and anti-α-internexin were from Chemicon (Temecula, CA); anti-α-tubulin, pGEX-4T-1 vector, and BL21 bacteria were from Amersham Pharmacia Biotech; horseradish peroxidase-coupled goat anti-rabbit immunoglobulin IgG (H&L) was from Calbiochem (San Diego, CA); horseradish peroxidase-coupled goat anti-mouse Ig (G + A + M) was from Cappel (West Chester, PA); SuperSignal, SuperSignal ULTRA, and SuperSignal BLAZE chemiluminescent detection reagents for Western blotting were from Pierce; polyvinylidine difluoride membrane was from Millipore; and protein G plus/protein A-agarose was from Oncogene Research Products (Cambridge, MA). Peptide β (KGAAPGPPRHG) was chemically synthesized by Iwaki Glass (Funabashi, Japan). All other chemicals were of reagent grade.

RT-PCR—Total RNA was isolated from the rat tissues using ISOGEN according to the manufacture's instructions. The RNA was incubated with DNase I (Qiagen) at 37 °C for 30 min and treated by Isogen again. RT-PCR was essentially followed by the protocol of the RNA PCR kit (Avian Myeloblastosis Virus) version 2.1. One microgram

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB016962.

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[1] The abbreviations used are: PSD, postsynaptic density; anti-α1, anti-synGAP-α1 antibody; anti-β, anti-synGAP-β antibody; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; GAP, GTPase-activating protein; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; RT, reverse transcriptase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

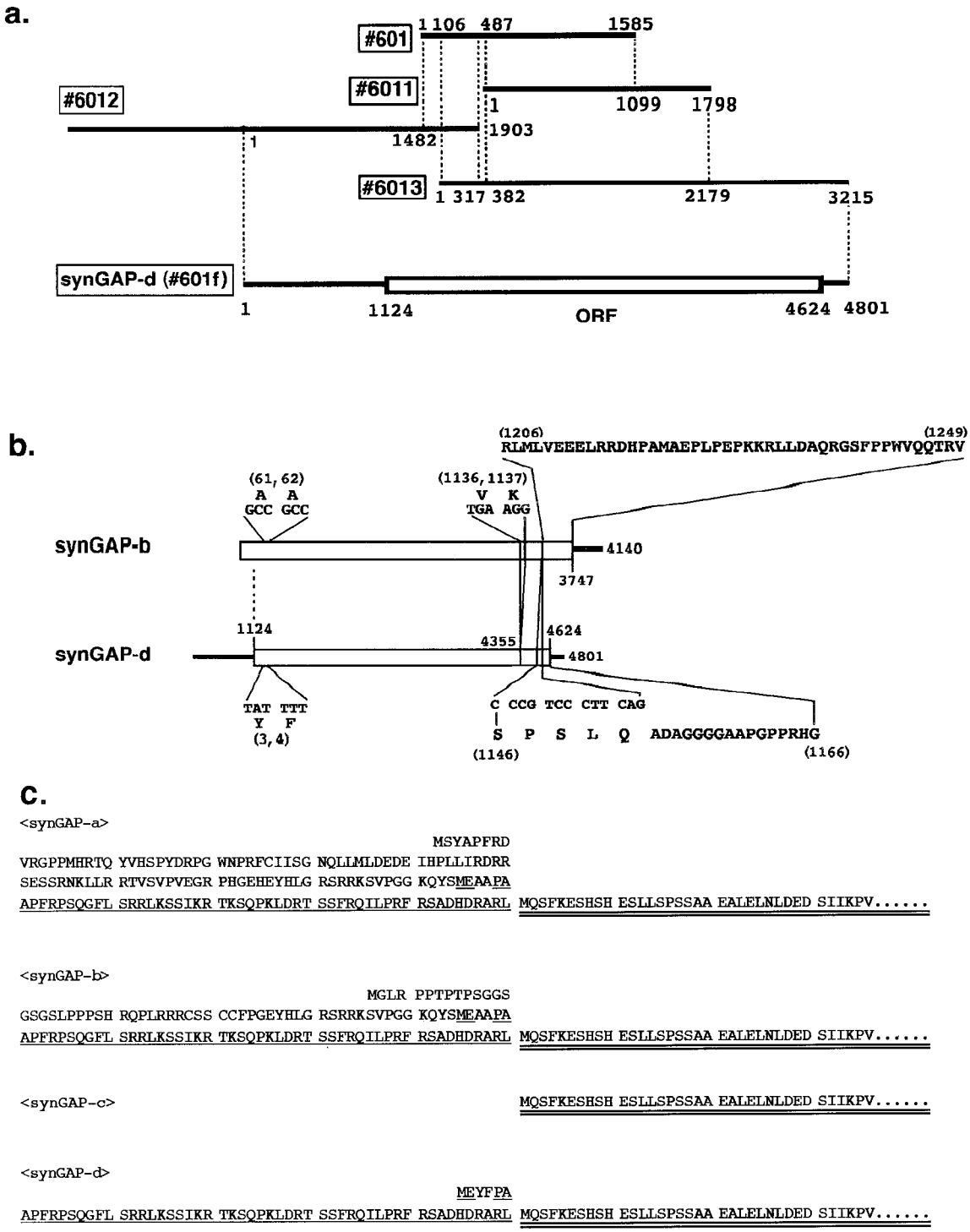

FIG. 1. **Cloning of a synGAP-d gene.** *a*, isolated clones with a partial sequence of synGAP-d. *b*, comparison of synGAP-b and synGAP-d. Structures of synGAP-b and synGAP-d DNAs are illustrated, and the differences in the nucleotide sequences and amino acid sequences are indicated. *c*, N-terminal amino acid sequences of various synGAP isoforms are shown. The sequences common to all the isoforms and those common to the a, b, and d isoforms are *double-underlined* and *underlined*, respectively. *ORF*, open reading frame.

of total RNA was used for the RT-PCR reaction. cDNA was produced at 42 °C for 30 min, and the following PCR reaction was 28 cycles, unless stated otherwise, of 30 s of 94 °C, 30 s of 60 °C, and 1 min of 72 °C. We tested 7–35 cycles of the PCR and confirmed that the quantity of amplified synGAP gene products did not reach the saturating level at the end of 28 cycles of the reaction. PCR products were separated by electrophoresis using 6% polyacrylamide gel and visualized by ethidium bromide. The ethidium bromide-stained images were printed by a CCD camera system (ATTO printograph, ATTO Bioscience & Technology, Tokyo, Japan).

The two sets of PCR primers spanning the alternatively spliced junction in the 3′ tail portion of the synGAP were synthesized. Primer set 1 corresponded to nucleotides 4500–4519 (5′ primer, 5′-AGAAGCGCTTGAGGCAGCAG-3′) and 4658–4639 (3′-primer, 5′-GTCGAGCAGCCTCTTCTTGG-3′) of the synGAP-d cDNA, and primer set 2 corresponded to nucleotides 4319–4339 (5′ primer, 5′-TACTCGAAGTCCATGGACGAG-3′) and 4707–4687 (3′ primer, 5′-TCACACGCGGGTTTGTTGGAC-3′) of the synGAP-d cDNA. Primers for glyceraldehyde-3-phosphate dehydrogenase were 5′-GCTGTGGGCAAGGTCATC-3′ and 5′-CCTGGTCCTCAGTGTAGC-3′ (sense and antisense, respectively); those for rat ninjurin2 (GenBank™ accession number AF250322) were 5′-ATGGAGTCAGACCGAGAAATC-3′ and 5′-AGCCGCATGGCATTGGAC-3′

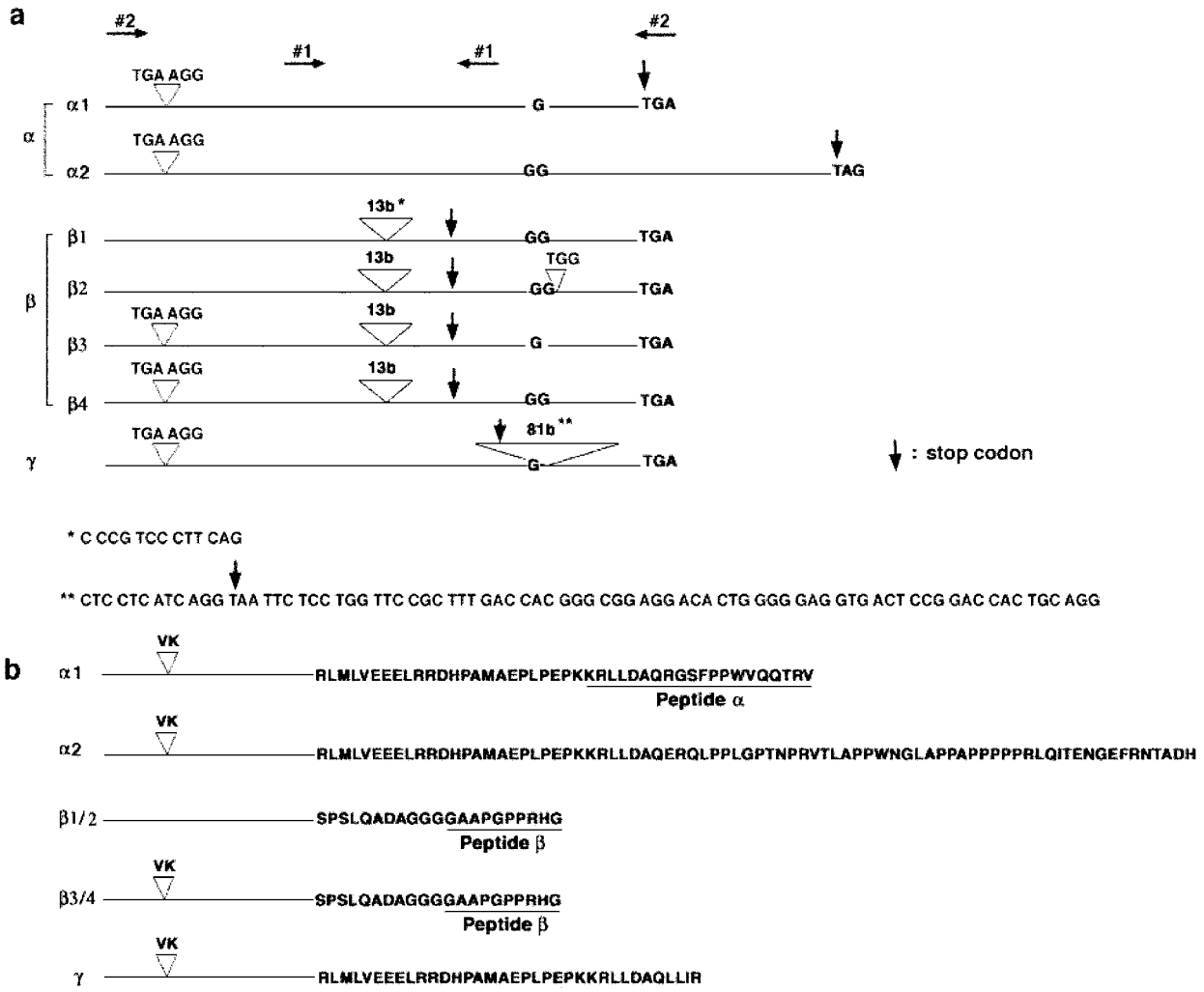

FIG. 2. **A schematic diagram showing the different nucleotide and amino acid sequences of synGAP C-terminal variants.** *a*, comparison of nucleotide sequences of the synGAP mRNA isoforms. Only the 3′ portions are shown (compare with Fig. 1*b*). *Vertical arrows* indicate the positions of stop codons. *#1* and #2 refer to position of primer sets 1 and 2. *b*, comparison of amino acid sequences of synGAP C-terminal isoforms. Only the C-terminal portions are shown. The C-terminal sequence of the β isoform is the same as that in the p135synGAP C-terminal variant (1). *Peptide α* and *Peptide β* indicate the sequences that were used to produce anti-α1 and anti-β, respectively. Peptide β was also used for the pull-down assay.

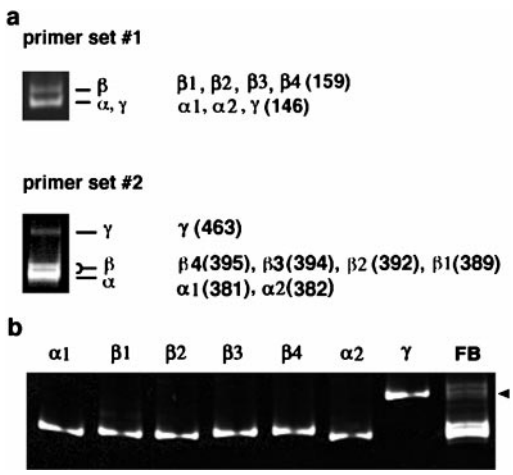

FIG. 3. **Separation of alternatively spliced variants of synGAP cDNA by polyacrylamide gel electrophoresis.** *a*, RT-PCR using RNA isolated from the rat forebrain as a template. The lengths (base number) of the isoforms are shown in *parentheses*. *b*, various synGAP cDNA clones subcloned into pGEM-easy T vector were digested with *Eco*RI and separated in polyacrylamide gel. The lane marked *FB* shows the RT-PCR products from forebrain mRNA using primer set 2. An *arrowhead* indicates the γ band in the forebrain sample.

(sense and antisense, respectively).

Construction and Expression of the GST Fusion Protein—cDNAs corresponding to amino acid residues 729–1166 of synGAP-d protein, amino acid residues 787–1249 of synGAP-b protein, the C-terminal 21-amino acid portion of synGAP-β, the C-terminal 48-amino acid portion of synGAP-α2, and the C-terminal 35-amino acid portion of synGAP-γ were subcloned into pGEX-4T-1 vector. The constructs were transfected into BL21 cells, and transcription was induced by isopropyl-1-thio-β-D-galactopyranoside. The whole cell lysates were analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250 or Western blotting.

Generation of Antibodies—Antiserum specific to PSD protein was produced by immunizing a rabbit using whole PSD proteins as immunogen after sonicating the protein to maximally unfold the PSD structures.

Peptide β, the C-terminal peptide of synGAP-β with N-terminally added lysine (KGAAPGPPRHG), was immobilized to keyhole limpet hemocyanine and used to immunize a rabbit to generate antiserum. Anti-synGAP-β antibody (anti-β) was affinity-purified by the peptide β immobilized to Affi-Gel 10. Anti-CaMKII antibody and anti-phosphorylated CaMKII peptide antibody were produced as reported previously (14, 15).

Immunoprecipitation and Western Blotting—PSD proteins were solubilized at 4 °C for 2 h in a low stringent buffer (16) or at 4 °C for 30 min in 50 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl, 2.7 mM KCl, 5 mM EDTA, 5 mM EGTA, 2% SDS, 50 mM NaF, 100 μM sodium vanadate, and protease inhibitor mixture (solubilization buffer). In the

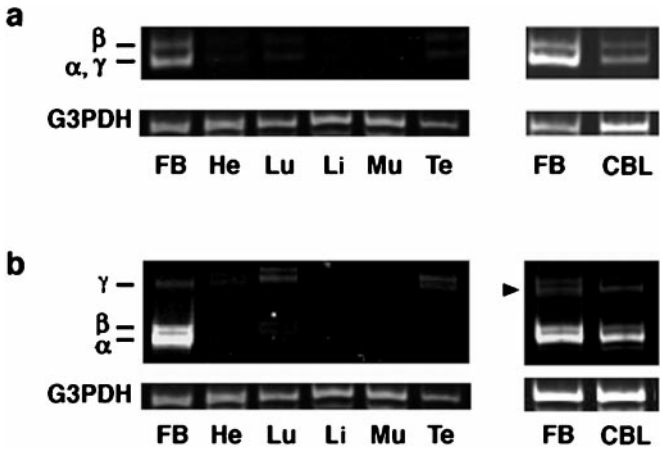

FIG. 4. **Tissue distribution of synGAP isoforms revealed by RT-PCR.** RT-PCR was performed using primers sets 1 (*a*) and 2 (*b*). The total RNA was isolated from forebrain (*FB*), cerebellum (*CBL*), heart (*He*), lung (*Lu*), liver (*Li*), skeletal muscle (*Mu*), and testis (*Te*). An *arrowhead* indicates the position of the γ band. *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase.

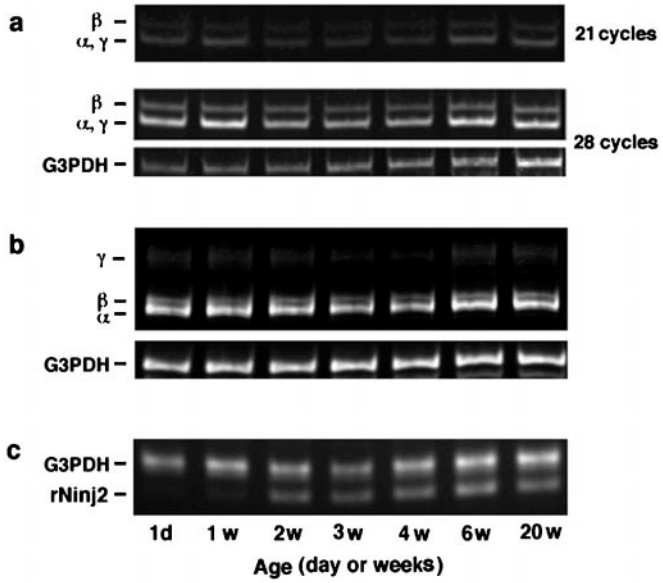

FIG. 5. **Developmental changes in the synGAP isoform expression revealed by RT-PCR.** RT-PCR was performed using primer set 1 (*a*), primer set set 2 (*b*), and primers for rat ninjurin (rNinj2, GenBank™ accession number AB016962) (*c*), followed by acrylamide (*a* and *b*) or agarose (*c*) gel electrophoresis. Total RNA was prepared from the rat forebrain at postnatal day 1 (*1d*) through 20 weeks (*1w*, *2w*, *3w*, *4w*, *6w*, and *20w*). The numbers of PCR cycles are shown on the *right* in *a*, and 28 cycles were used for *b* and *c*. *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase.

latter case, five volumes of dilution buffer containing 2% Triton X-100 (17) were further added and incubated at 4 °C for an additional 2 h. The supernatant was obtained after centrifugation at 10,000 rpm for 10 min, incubated at 4 °C for 3 h or overnight with antibodies, and then mixed for 2 h with protein G plus/protein A-agarose. Alternatively, the supernatant was incubated with antibody immobilized chemically to protein G plus/protein A-agarose. The gel was pelleted and washed three times, and immunoprecipitated proteins were separated by SDS-PAGE.

For Western blotting, proteins separated by SDS-PAGE were electroblotted onto polyvinylidine difluoride membranes. The membranes were blocked with 5% skimmed milk, incubated with antibodies at room temperature for 1 h, and immunostained using the chemiluminescent substrate. Chemiluminescence was captured and visualized with a CCD video camera system (Atto Densitograph Lumino CCD AE-6930, ATTO Bioscience & Technology, Tokyo, Japan).

Pull-down Assay—PSD proteins (20 μg) were solubilized by boiling for 5 min in the solubilization buffer (see above) containing 1 mM

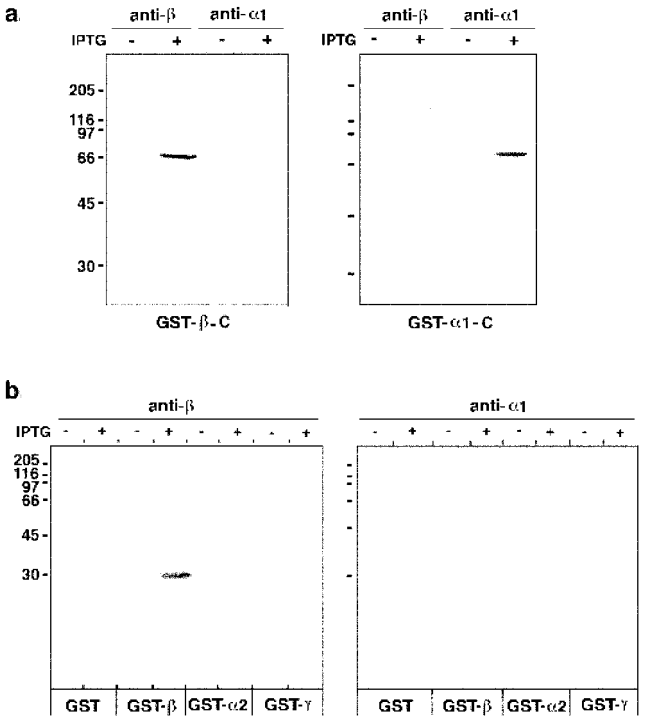

FIG. 6. **Specificity of the anti-α1 and anti-β.** *a*, GST fusion proteins were expressed in BL21 cells by induction with isopropyl-1-thio-β-D-galactopyranoside (*IPTG*), and whole lysates were Western blotted by anti-α1 or anti-β. GST-β-C and GST-α1-C contain amino acid residues 729–1166 of synGAP-dβ1 and amino acid residues 787–1249 of synGAP-b (α1 form), respectively. *b*, GST fusion proteins containing various isoforms were expressed, and whole lysates were Western blotted by anti-β or anti-α1. GST-β, GST-α2, and GST-γ contain the C-terminal 21 amino acids of the β isoform, the C-terminal 48 amino acids of the α2 isoform, and the C-terminal 35 amino acids of the γ isoform, respectively. Molecular masses are shown on the *left* in kDa.

dithiothreitol, five volumes of Triton X-100-containing dilution buffer were added, and the mixture incubated at 4 °C for 2 h. Supernatant was obtained by centrifugation at 10,000 rpm for 15 min and incubated at 4 °C for 2 h with peptide β coupled to Affi-Gel 10 (bed volume, 5 μl). The gel was washed four times by brief centrifugation and aspiration. The pull-down proteins separated by SDS-PAGE were analyzed by silver staining or Western blotting.

Cell Culture and Cell Staining—Whole cerebral neocortices of embryonic day 18 rats (Sprague-Dawley) were mechanically dissociated and plated onto poly-D-lysine-coated dishes at a lower density (500–800 cells/mm^2). Cortical neurons were grown with Dulbecco's modified Eagle's medium containing 0.5 mM pure glutamine (Ajinomoto, Tokyo, Japan), 2% fetal bovine serum, nutrient mixture N2, and 10 mM Hepes (pH 7.3) (18). The following day the original medium was replaced by the medium lacking serum (serum-free N2 medium). Cells (15 days on culture) were fixed with 4% paraformaldehyde as described previously (18) and stained with rabbit anti-β, anti-PSD-95, or anti-synaptophysin antibody combined with fluorescently labeled secondary antibodies. They were examined and photomicrographed with a Zeiss Axioskop microscope.

Subcellular Fractionation—Synaptic plasma membrane fraction of the forebrain was prepared from Wistar rats (6 weeks old, male) by the method of Cohen *et al.* (19) with modification by Wu *et al.* (20), and the PSD fraction was successively prepared as described previously (14, 21). The postsynaptic raft (or dendritic raft) fraction (22) was obtained as a band between 0.32 and 1.0 M sucrose after sucrose gradient centrifugation to obtain the PSD fraction. P1 (fraction containing nuclei and cell debris), P2 (crude mitochondrial fraction), and Syn (synaptosomal fraction) were obtained during PSD isolation. Protein from these fractions was assayed using the method of Lowry *et al.* (23) with bovine serum albumin as the standard. All preparations were stored at −80 °C until use.

RESULTS

Molecular Cloning of synGAP cDNA—Antiserum specific to PSD protein was produced by immunizing a rabbit using whole

PSD protein as the immunogen. The antibody recognized a number of proteins in the PSD fraction and relatively few proteins in the other subcellular fractions prepared from the brain, and it did not cross-react with Triton X-100-insoluble cytoskeletal proteins of other tissues such as the liver (data not shown). We used this antibody for the initial screening of the cDNA library. About 500,000 plaques of the λZAP cDNA library were plated and screened at first, and we obtained five different clones, of which three DNA sequences were identical to the fodrin α subunit, plectin, and neurofilament H subunit, and one of which, clone 601, had not previously been reported in the initial data base. We proceeded with the cloning using clone 601 cDNA as a probe and obtained three independent cDNAs, 6011, 6012, and 6013. Finally, we obtained the sequence of 601f by compiling the 601, 6011, 6012, and 6013 clones (Fig. 1*a*).

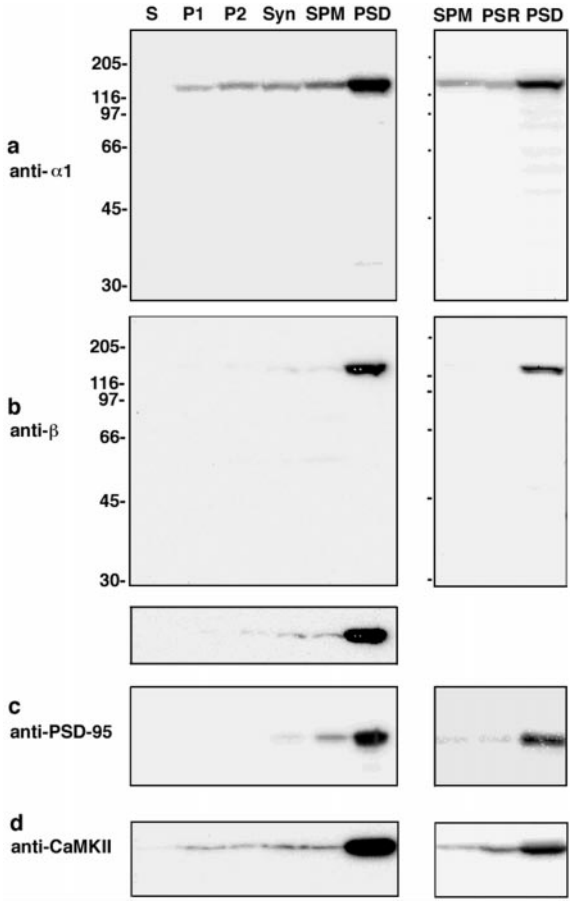

FIG. 7. **Localization of the α1 and β isoforms in the various subcellular fractions.** Proteins (20 μg) of various fractions prepared from the rat forebrain were separated on 10% polyacrylamide gel and detected with anti-α1 (*a*), anti-β (*b*), anti-PSD-95 (*c*), or anti-CaMKII (*d*). The *lower panel* of *b* shows a picture taken at higher sensitivity. *S*, *Syn*, and *PSR* refer to soluble, synaptosome, and postsynaptic raft (22) fractions, respectively. Molecular masses are shown on the *left* in kDa. Only the regions close to PSD-95 or CaMKII band are shown in the *lower two gels* because of space limits.

601f DNA was 4801 bases long and contained a 3501-base open reading frame (1124–4624). The nucleotide sequence of the coding region had a >99% identity in >1092 amino acid overlaps with those of various types of synGAPs. Therefore, we named the clone synGAP-d (GenBank™ accession number AB016962). The synGAP-d protein most resembled synGAP-b (GenBank™ accession number AF058790), and the differences between the two proteins are illustrated in Fig. 1 (*b* and *c*). SynGAP-b protein was 58 amino acids longer than synGAP-d in the N terminus (Fig. 1*c*). Ala^{61} and Ala^{62} in the synGAP-b were replaced by Tyr and Phe, respectively, in the synGAP-d. SynGAP-d had deletions of two amino acids, Val^{1136} and Lys^{1137}, shown in synGAP-b. SynGAP-d mRNA had an insertion of 13 nucleotides near the 3′ tail of the coding sequence, which made a frameshift, producing a C-terminal tail completely different from that of synGAP-b (Fig. 1*b*). A splice variant with this 13-base nucleotide had also been reported in p135synGAP by Chen *et al.* (1) (GenBank™ accession number AF055883). The C-terminal of the synGAP-d lacked the (S/T)*X*V motif, an association motif with PSD-95. The synGAP-d had a pleckstrin homology domain, a C2 domain, a GAP domain, and a Pro-rich domain as found in other synGAP proteins.

Identification of synGAP mRNA by RT-PCR and Classification of synGAP Isoforms—The presence of synGAP-b and synGAP-d mRNAs was confirmed by RT-PCR in the total RNA prepared from the rat forebrain. Two primer sets, sets 1 and 2, used for the RT-PCR were located surrounding the 13-oligonucleotide insert in the synGAP-d (Fig. 2*a*). RT-PCR using primer sets 1 and 2 produced two and three major bands, respectively (Fig. 3*a*). We purified these bands by extracting the DNA from the cut bands, subcloned them into pGEM-T easy vector (Fig. 3*b*), and determined their nucleotide sequences. Unexpectedly, we obtained seven different sequences with minor differences. These seven synGAP clones of 3′ tail coding sequences were classified based on sequence similarity and named systematically as synGAP-α (α1 and α2), β (β1, β2, β3, and β4), and γ, as

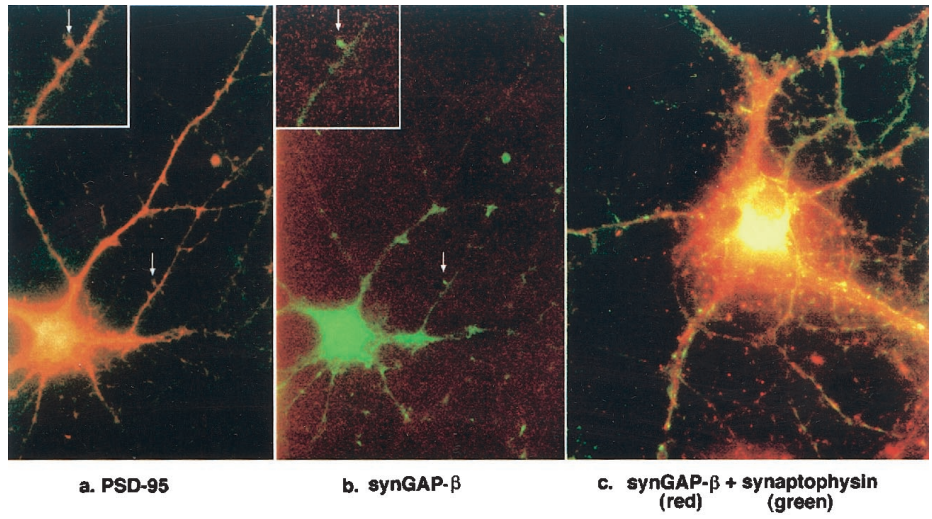

FIG. 8. **Localization of the β isoform in cultured cortical neurons.** Subcellular localization of the novel synGAP isoform was examined in the cultured cerebral neurons. PSD-95 (*a*) and the β isoform (*c*) were finally labeled with Texas red (*red*), and synGAP-β (*b*) and synaptophysin (*c*) were labeled with fluorescein isothiocyanate (*green*). *Insets* in *a* and *b* show the area around a typical spine indicated by *arrows*.

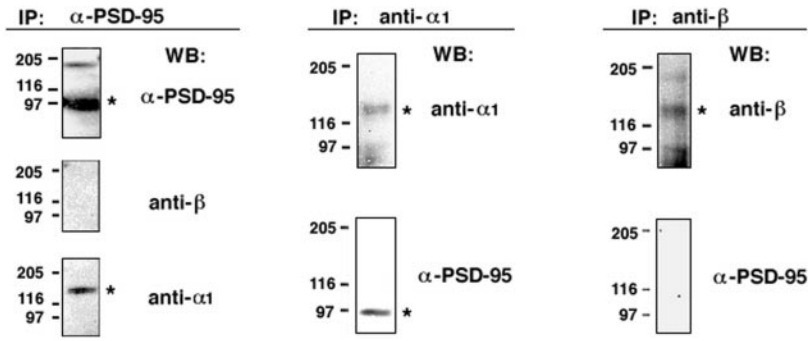

FIG. 9. **Co-immunoprecipitation of synGAP isoforms with PSD-95.** PSD proteins (300 μg) were solubilized at 4 °C for 2 h with low stringent buffer and then immunoprecipitated with either anti-PSD-95 (*α-PSD-95*) antibody, anti-α1, or anti-β. Western blotting was carried out using various antibodies shown on the *right*. The target protein bands for Western blotting are indicated by *asterisks*. *IP* and *WB* refer to immunoprecipitation and Western blotting, respectively.

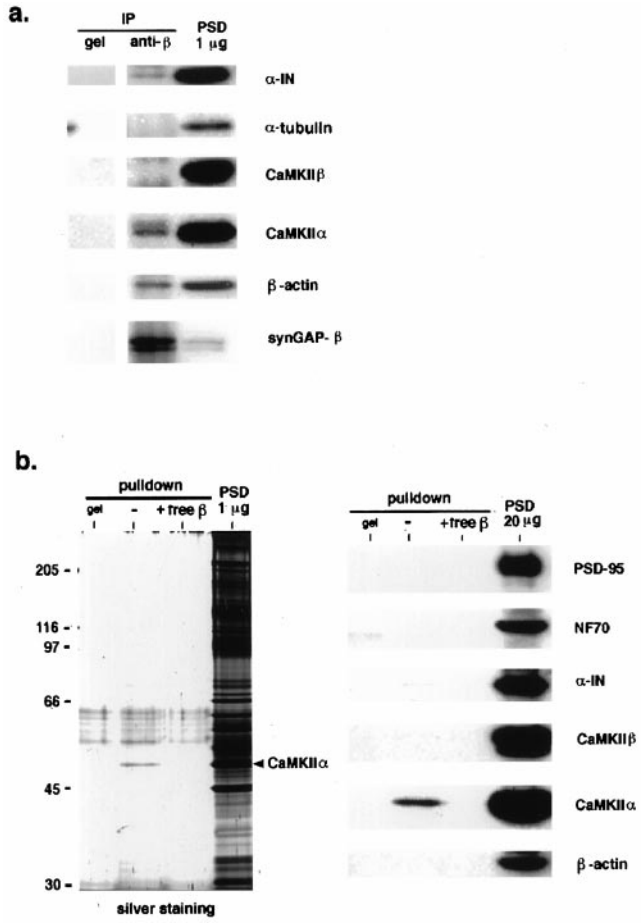

FIG. 10. **Survey of the β isoform-binding proteins by co-immunoprecipitation and pull-down assays.** *a*, PSD proteins (20 μg) were solubilized in a solubilization buffer containing 2% SDS at 4 °C for 30 min, five volumes of 2% Triton X-100 were added, and the mixture was immunoprecipitated (*IP*) with anti-β immobilized chemically to protein G plus/protein A-agarose. Protein G plus/protein A-agarose without a linked antibody was used as a control. Western blotting was carried out using various antibodies. Target proteins for Western blotting are shown on the *right*. Immunoprecipitated and control materials were processed simultaneously under similar conditions. *b*, PSD proteins (20 μg) were solubilized for 5 min by boiling in the solubilization buffer containing 2% SDS and 1 mM dithiothreitol, five volumes of 2% Triton X-100 were added, and the mixture was incubated with peptide β (C-terminal 10-amino acid peptide with N-terminally added lysine; see Fig. 1*b*) immobilized to Affi-Gel 10. Pull-down material was separated in polyacrylamide gel and stained with silver or immunoblotted with various antibodies specific to the proteins shown on the *right*. *α-IN* and *NF70* refer to α-internexin and neurofilament of 70 kDa, respectively.

shown in Fig. 2*a*. We omitted "d" from the names, because this classification might also be used in other synGAP isoforms in addition to synGAP-d. The presence of synGAP-α2, which was longer than the end point of the 3′ primer, was confirmed by RT-PCR using a different 3′ primer, which hybridized to the far 3′ portion (data not shown), and was supported by the finding that synGAP-α2 produced a C-terminal tail with 11 amino acid sequences extremely similar to those of human RasGAP and nGAP (GenBank™ accession number AL035702 and AF047711, respectively). The single base g insertion seen in the α2, β1, β2, and β4 may not be an artificial by-product produced by PCR, because this insertion was found in the 6013 clone. SynGAP-a, -b, and -c, with common C-terminal sequences, can also be named as synGAP-aα1, synGAP-bα1, and synGAP-cα1, respectively, when adopting the present classification.

These seven synGAP mRNAs encoded five different synGAP protein isoforms (Fig. 2*b*). There are at least four synGAP proteins in which the C-terminal tails were different. The C-terminal (S/T)*X*V motif, a PDZ-binding motif, was present only in the synGAP-α1 protein. All of the β isoform proteins showed the same C-terminal tail sequence, which was the same as that found in the C terminus of p135synGAP (1).

Tissue Distribution and Developmental Changes in the Expression of synGAP mRNA Variants—Expression of synGAP variants was examined in various tissues by RT-PCR using two different primer sets (Fig. 4). All of the synGAP variants were specifically expressed in the brain. The expression of the γ form was extremely low compared with those of α and β, and bands close to but slightly larger than the γ band were detected in the PCR of non-neuronal tissues. Expression profiles of synGAP-α and β were similar between the forebrain and cerebellum, whereas the expression levels of both genes in the cerebellum were lower than those in the forebrain.

Developmental changes in the expression of synGAP variants were examined in the rat forebrain by RT-PCR (Fig. 5). The level of synGAP mRNAs was as high as the adult level 1 day and 1 week after birth. The assay condition was judged to be appropriate because expression of the nerve injury-induced protein (ninjurin) (24, 25) assayed under the same conditions showed developmental change with a gradual increase, saturating at 6 weeks after birth. Amplified synGAP gene products did not reach the saturating level at the end of 28 cycles of PCR.

Cellular Distribution of synGAP-β—Before using anti-β in immunochemical and immunocytochemical studies, we first tested the specificity of the antibody by Western blotting (Fig. 6). Anti-β reacted specifically with the expressed GST-synGAP-β1 C-terminal half protein but not with the expressed GST-C-terminal half synGAP-α protein (Fig. 6*a*). The antibody also reacted with the GST 21-amino acid C-terminal peptide of synGAP-β but not with GST itself, the GST-α2 C-terminal peptide, nor the GST-γ C-terminal peptide (Fig. 6*b*). Thus, the anti-β specifically reacted with the C-terminal tail portion of synGAP-β. Anti-synGAP antibody purchased from Upstate Biotechnology, Inc. (termed here as anti-α1) reacted with the GST C-terminal half synGAP-α1 protein but not with the GST C-terminal half synGAP-β protein, the GST-α2-C-terminal

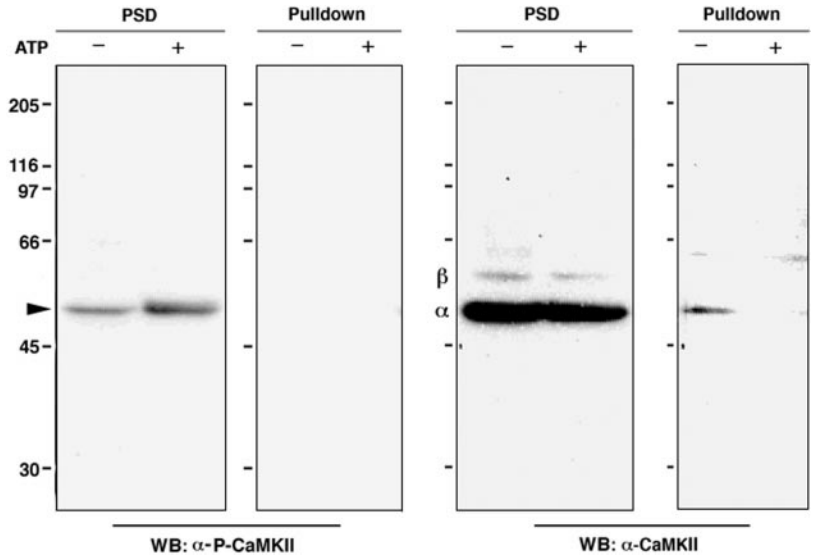

FIG. 11. **Effect of CaMKII autophosphorylation on the CaMKII α subunit binding to peptide β.** PSD proteins (20 μg) were incubated at 25 °C for 10 min with Ca^{2+} and calmodulin either in the presence or absence of 500 μM of ATP, and the reaction was terminated with solubilization buffer for SDS-PAGE containing SDS and mercaptoethanol. The PSD proteins were processed for the pull-down assay as described in the legend for Fig. 10*b*. Western blotting was done using either anti-phosphorylated CaMKII peptide antibody (α-*P-CaMKII*) or anti-CaMKII antibody (α-*CaMKII*). The *lanes* marked as *PSD* show the Western blotting of PSD proteins (20 μg) that were incubated in the same way but without pull-down treatment. The *arrowhead* indicates the P-CaMKII α subunit, and α and β indicate the α and β subunits of CaMKII, respectively. *WB* refers to Western blotting.

peptide, nor the GST-γ C-terminal peptide (Fig. 6). Thus, these antibodies reacted mutually exclusively and specifically to synGAP-α1 and -β, respectively.

Then we examined the subcellular distribution of synGAP-α1 and -β (Fig. 7). Both antibodies detected bands in the 130 kDa region that were considered to be synGAP proteins. The distribution of synGAP-β was highly restricted in the PSD fraction, which was confirmed by the picture taken at higher sensitivity (Fig. 7*b*, *lower panel*). SynGAP-α1 was also highly enriched in the PSD but was also detected, although the content was low, in the other subcellular fractions: postsynaptic (or dendritic) raft, synaptic plasma membrane, synaptosome, and P2 and P1 fractions. The distribution patterns of PSD-95 and the CaMKII α subunit verified the subcellular fractionation, with both proteins being enriched in the PSD fraction.

Next we examined the intracellular distributions of the synGAP-α1 and -β in cultured neurons (Fig. 8). Immunoreactivity for synGAP-β was localized in the dendrites including spines and co-localized with PSD-95, a postsynaptic marker in the forebrain (26). Their co-localization in the spines was shown typically in Fig. 8 (*insets* in *a* and *b*). The distribution of synGAP-β and synaptophysin, a presynaptic marker (27), was compared by double immunofluorescent staining (Fig. 8*c*). Synaptophysin immunoreactivity was distributed in a patch-like pattern and localized in close proximity to synGAP-β. Patchy stainings of synaptophysin are believed to be localization of presynaptic terminals. Thus, the postsynaptic localization of synGAP-β was indicated by immunostaining of cultured cortical neurons as well.

Identification of synGAP-β-binding Protein—SynGAP-α1 possessed the C-terminal (S/T)*X*V motif, which was suggested to bind to the first or second PDZ domain of the PSD-95 protein (28). Therefore, synGAP-α1 is suggested to be anchored to PSD with intervention by PSD-95. Unexpectedly, synGAP-β was more restricted to the PSD fraction than synGAP-α1, although synGAP-β lacked the PSD-95-binding motif (Fig. 7). This finding suggested searching the synGAP-β-binding protein(s) in the PSD proteins.

We first tested the interaction of the synGAP-β with PSD-95 in the PSD by co-immunoprecipitation assay (Fig. 9). SynGAP-α1, but not synGAP-β, was co-precipitated when PSD proteins were immunoprecipitated with anti-PSD-95 antibody. In reverse, PSD-95 was co-precipitated when PSD proteins were immunoprecipitated with anti-α1 but not when they were immunoprecipitated with anti-β.

Next we surveyed the binding protein(s) by a co-immunoprecipitation study (Fig. 10*a*). In this case, we adopted the solubilization method of Lau *et al.* (17), because this method enabled the efficient solubilization of PSD proteins and also the maintenance, in part, of the undissociated functional protein complexes. In fact, this method solubilized PSD proteins nearly completely (data not shown). Thus, this solubilization method was judged to be the most appropriate for the immunoprecipitation of PSD proteins. We used antibody immobilized chemically to agarose gel, because otherwise immunoglobulin heavy and light chains hindered detection of some proteins in the polyacrylamide gel. We tested some cytoskeletal proteins and PSD-enriched proteins by Western blotting. The CaMKII α subunit, β-actin, and α-internexin were co-immunoprecipitated with synGAP-β. The amounts of these co-immunoprecipitated proteins were low compared with synGAP-β, probably because of dissociation of the functional complexes, to some extent, after solubilization of PSD proteins with SDS. These proteins were not precipitated when using only protein G plus/protein A-agarose gel that was treated in a similar way to immobilize the antibody to the gel. Thus, these precipitations were judged to be specific. The α-tubulin and CaMKII β subunit were not co-immunoprecipitated with synGAP-β.

Finally we searched the direct binding partner using the pull-down method (Fig. 10*b*). When solubilizing, PSD proteins were completely dissociated to monomeric conditions by boiling in the presence of SDS and dithiothreitol (17). This method enabled the detection of a direct binding partner alone. We first detected the pull-down materials separated in a polyacrylamide gel by silver staining and found that only one 50-kDa protein, comigrating with the CaMKII α subunit in the PSD fraction, was pulled down (Fig. 10*b*, *left panel*). We then confirmed the pull-down material as a CaMKII α subunit by Western blotting. α-Internexin and β-actin were not detected, which was different from the findings of the co-immunoprecipitation study (Fig. 10*a*). The CaMKII β subunit was not bound to peptide β. The binding of the CaMKII α subunit was judged to be specific because the band was not observed when using only Affi-Gel or when free peptide β (500 μg/tube) was added during the pull-down.

We next examined the effect of CaMKII autophosphorylation on the CaMKII α subunit binding to peptide β. CaMKII in the PSD fraction was autophosphorylated in the presence of Ca^{2+} and calmodulin and incubated with peptide β immobilized to Affi-Gel 10, as shown in Fig. 10*b*. Autophosphorylation of the CaMKII was confirmed by Western blotting using anti-phos-

phorylated CaMKII peptide antibody (Fig. 11, *PSD panels*). The autophosphorylated CaMKII α subunit was not pulled down by peptide β, under similar conditions where the non-phosphorylated CaMKII α subunit was pulled down (Fig. 11, *Pulldown panels*).

DISCUSSION

We have cloned a novel gene, synGAP-d, that encodes a synGAP isoform with an unique C-terminal 21-amino acid sequence. The cloned synGAP-d was most similar to synGAP-b except for the N-terminal and C-terminal tail sequences. SynGAP-d protein is 58 amino acids shorter than synGAP-b in the N terminus and has a replacement near the N terminus, and synGAP-d DNA has a deletion of six nucleotides near the C-terminal portion of the protein and an insertion of 13 nucleotides in the 3′ portion of its coding sequence; this causes a frameshift and produces a 21-amino acid-long C-terminal sequence unique to the synGAP-d.

We also found that there are various synGAP isoforms of which the C-terminal portions are unique. Three isoforms of the synGAP protein with different N-terminal sequences have been reported: synGAP-a (identical to p135synGAP), -b, and -c (1, 2) (Fig. 1*c*). p135synGAP possesses putative splice variants with insertions in the N-terminal or C-terminal region (1). The present finding increases the repertoire of the synGAP protein isoforms.

We classified and named the synGAP isoforms identified in this study based on the types of differences (Fig. 2). We omit "a," "b," "c," or "d" from the names. All these a, b, and c isoforms are N-terminal variants as shown in Fig. 1*c*, whereas the C-terminal sequences are common to all of these variants. Variation in the C terminus found in synGAP-d may exist in combination with these N-terminal variations, because this C-terminal variation has also been reported in p135synGAP (1) and synGAP-d, and anti-β-immunoreactive bands with slightly different molecular sizes were detected as shown in Fig. 10*a*. Heterogeneity of the synGAP proteins may add differential roles to these proteins in various brain regions.

Furthermore we identified a specific binding protein for the synGAP-β isoform as a CaMKII α subunit. The C-terminal portion of synGAP proteins is important, especially for their targeting to specific sites in the neuronal cells, because previously reported synGAP possesses the (S/T)*X*V motif in the C-terminal end, and the protein interacts with the first or second PDZ domain of PSD-95 via this motif. Thus, the (S/T)*X*V motif-containing synGAPs can be localized to PSD through this interaction. The difference in the binding partners between the (S/T)*X*V motif-containing synGAP and the synGAP-β form may explain the difference in their subcellular distribution. Unexpectedly, the (S/T)*X*V motif-lacking β isoform was more restricted to the PSD fraction than the (S/T)*X*V motif-containing synGAP, which suggested that there should be a specific binding molecule in the PSD fraction. In fact, the unique synGAP-β C-terminal sequence was found to bind to the CaMKII α subunit, which was a well known PSD protein (14). Therefore, synGAP-β isoforms may be concentrated in the PSD fraction through binding to the CaMKII α subunit. The interaction between the CaMKII α subunit and the synGAP$-\beta$ form could be regulated by synaptic activity, because peptide β did not interact with the autophosphorylated α subunit of CaMKII (Fig. 11).

The present findings, together with previous observations (1, 2), suggest that both the (S/T)*X*V motif-containing synGAP and the β isoform are localized in the postsynaptic sites including spines. Close interaction of synGAP-β with the CaMKII α subunit may have specific functional significance in addition to that as a substrate for CaMKII. The functional significance of this interaction awaits further study.

REFERENCES

1. Chen, H.-J., Rojas-Soto, M., Oguni, A., and Kennedy, M. B. (1998) *Neuron* **20,** 895–904
2. Kim, J. H., Liao, D., Lau, L.-F., and Huganir, R. L. (1998) *Neuron* **20,** 683–691
3. Suzuki, T., Okumura-Noji, K., and Nishida, E. (1995) *Neurosci. Res.* **22,** 277–285
4. Suzuki, T., Mitake, S., and Murata, S. (1999) *Brain Res.* **840,** 36–44
5. Manabe, T., Aiba, A., Yamada, A., Ichise, T., Sakagami, H., Kondo, H., and Katsuki, M. (2000) *J. Neurosci.* **20,** 2504–2511
6. Orban, P. C., Chapman, P. F., and Brambilla, R. (1999) *Trends Neurosci.* **22,** 38–44
7. Impey, S., Obrietan, K., and Storm, D. R. (1999) *Neuron* **23,** 11–14
8. Winder, D. G., Martin, K. C., Muzzio, I. A., Rohrer, D., Chruscinski, A., Kobilka, B., and Kandel, E. R. (1999) *Neuron* **24,** 715–726
9. Narisawa-Saito, M., Silva, A. J., Yamaguchi, T., Hayashi, T., Yamamoto, T., and Nawa, H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2461–2466
10. Levine, E. S., Dreyfus, C. F., Black, I. B., and Plummer, M. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8074–8077
11. Kang, H., Welcher, A. A., Shelton, D., and Schuman, E. M. (1997) *Neuron* **19,** 653–664
12. Brambilla, R., Gnesutta, N., Minichiello, L., White, G., Roylance, A. J., Herron, C. E., Ramsey, M., Wolfer, D. P., Cestari, V., Rossi-Arnaud, C., Grant, S. G., Chapman, P. F., Lipp, H. P., Sturani, E., and Klein, R. (1997) *Nature* **390,** 281–286
13. Guo, H. F., Tong, J., Hannan, F., Luo, L., and Zhong, Y. (2000) *Nature* **403,** 895–898
14. Suzuki, T., Okumura-Noji, K., Tanaka, R., and Tada, T. (1994) *J. Neurochem.* **63,** 1529–1537
15. Suzuki, T., Okumura-Noji, K., Ogura, A., Kudo, Y., and Tanaka, R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 109–113
16. Suen, P. C., Wu, K., Xu, J. L., Lin, S. Y., Levine, E. S., and Black, I. B. (1998) *Mol. Brain Res.* **59,** 215–228
17. Lau, L.-F., Mammen, A., Ehlers, A. D., Kindler, S., Chung, W. J., Garner, C. C., and Huganir, R. L. (1996) *J. Biol. Chem.* **271,** 21622–21628
18. Narisawa-Saito, M., Carnahan, J., Araki, K., Yamaguchi, T., and Nawa, H. (1999) *Neuroscience* **88,** 1009–1014
19. Cohen, R. S., Blomberg, F., Berzins, K., and Siekevitz, P. (1977) *J. Cell Biol.* **74,** 181–203
20. Wu, K., Carlin, R., and Siekevitz, P. (1986) *J. Neurochem.* **46,** 831–841
21. Suzuki, T., Usuda, N., Ishiguro, H., Mitake, S., Nagatsu, T., and Okumura-Noji, K. (1998) *Mol. Brain Res.* **61,** 69–77
22. Suzuki, T., Ito, J., Takagi, H., Saitoh, F., Nawa, H., and Shimizu, H. (2001) *Mol. Brain Res.* **89,** 20–28
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
24. Araki, T., and Milbrandt, J. (1996) *Neuron* **17,** 353–361
25. Araki, T., and Milbrandt, J. (2000) *J. Neurosci.* **20,** 187–195
26. Garner, C. C., and Kindler, S. (1996) *Trends Cell Biol.* **6,** 429–433
27. Grabs, D., Bergmann, M., Schuster, T., Fox, P. A., Brich, M., and Gratz, M. (1994) *Eur. J. Neurosci.* **6,** 1765–1771
28. Songyang, Z., Fanning, A. S., Fu, C., Xu, J., Marfatia, S. M., Chishti, A. H., Crompton, A., Chan, A. C., Anderson, J. M., and Cantley, L. C. (1997) *Science* **275,** 73–77