THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 17, Issue of April 23, pp. 17980–17988, 2004

Printed in U.S.A.

Regulation of the Neuron-specific Ras GTPase-activating Protein, synGAP, by Ca^{2+}/Calmodulin-dependent Protein Kinase II*

Received for publication, December 23, 2003, and in revised form, February 11, 2004
Published, JBC Papers in Press, February 17, 2004, DOI 10.1074/jbc.M314109200

Jeong S. Oh, Pasquale Manzerra, and Mary B. Kennedy‡

From the Division of Biology 216-76, California Institute of Technology, Pasadena, California 91125

synGAP is a neuron-specific Ras GTPase-activating protein found in high concentration in the postsynaptic density fraction from mammalian forebrain. Proteins in the postsynaptic density, including synGAP, are part of a signaling complex attached to the cytoplasmic tail of the *N*-methyl-D-aspartate-type glutamate receptor. synGAP can be phosphorylated by a second prominent component of the complex, Ca^{2+}/calmodulin-dependent protein kinase II. Here we show that phosphorylation of synGAP by Ca^{2+}/calmodulin-dependent protein kinase II increases its Ras GTPase-activating activity by 70–95%. We identify four major sites of phosphorylation, serines 1123, 1058, 750/751/756, and 764/765. These sites together with other minor phosphorylation sites in the carboxyl tail of synGAP control stimulation of GTPase-activating activity. When three of these sites and four other serines in the carboxyl tail are mutated, stimulation of GAP activity after phosphorylation is reduced to 21 ± 5% compared with 70–95% for the wild type protein. We used phosphosite-specific antibodies to show that, as predicted, phosphorylation of serines 765 and 1123 is increased in cultured cortical neurons after exposure of the neurons to the agonist *N*-methyl-D-aspartate.

Storage of information in the brain is mediated in part by changes in the strength of synaptic connections between neurons initiated by specific patterns of electrical activity (1). These changes involve complex regulatory pathways that are controlled by the pattern of influx of Ca^{2+} ion through *N*-methyl-D-aspartate (NMDA)[1]-type glutamate receptors (NMDA receptors) at postsynaptic sites. Much present research concerns the nature of the relevant biochemical pathways and the mechanisms of Ca^{2+} control. One set of regulatory proteins associates tightly with the cytosolic portion of the NMDA receptor (2, 3). These include Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (4, 5), which is activated by Ca^{2+} flux through the receptor, and several proteins that are held near the receptor by the scaffold protein PSD-95 (6, 7). A complex containing many of these proteins can be isolated from brain homogenates and is called the postsynaptic density (PSD) fraction (8).

synGAP was identified as a prominent 140-kDa protein in the PSD fraction (9, 10) and as a protein that interacts with PSD-95 in a yeast two-hybrid screen (11). Its message is detected only in brain (10, 11). It is expressed only in neurons, including most excitatory neurons and a subset of inhibitory neurons (12), where it is highly localized to the postsynaptic density (10). It contains a PH domain, a C2 domain, and a Ras GAP domain that are 23%, 33%, and 47% similar, respectively, to those of the prototype Ras GAP protein p120 Ras GAP (13). In brain homogenates, synGAP is tightly bound to the particulate fraction and full-length synGAP has not yet been purified in soluble form; however, the GAP domain, expressed as a GST fusion protein in *Escherichia coli*, has been shown to stimulate hydrolysis of bound GTP by Ras (11). synGAP plays a crucial role in early development of the brain and in control of synaptic plasticity in the adult brain, as demonstrated by the phenotypes of mouse synGAP mutants (14, 15). Newborn mice with a deletion of synGAP die a few days after birth; whereas adult mice heterozygous for the deletion have altered synaptic plasticity (14).

In our first report of the characterization of synGAP, we showed that it is a prominent substrate for CaMKII in the postsynaptic density fraction and presented evidence that phosphorylation by CaMKII reduced its GAP activity ~50% (10). We later reported that this inhibition was an artifact caused by the combined presence of residual ATP from the phosphorylation reaction and the phosphatase inhibitor pyrophosphate in our Ras GAP assay (16). Here we have identified four of the principal phosphorylation sites for CaMKII in synGAP and re-examined the regulatory effect of their phosphorylation. We report that phosphorylation of these sites by CaMKII produces a 70–95% increase in the Ras GAP activity of synGAP. Because CaMKII is a major target of calcium influx through the NMDA receptor, this finding suggests that one result of activation of NMDA receptors at a synapse may be an increase in the rate of inactivation of Ras at that synapse. This mechanism might provide a means by which NMDA receptor activation modulates the action of receptor tyrosine kinases at excitatory synapses.

EXPERIMENTAL PROCEDURES

Materials—Acetonitrile, UV/HPLC grade, was purchased from EM Science (Gibbstown, NJ); HPLC/Spectra grade trifluoroacetic acid from Pierce (Rockford, IL); iodoacetamide from Sigma (St. Louis, MO); [γ-^{32}P]ATP and [α-^{32}P]GTP from ICN Pharmaceuticals Inc. (Irvine, CA); modified sequencing grade trypsin from Promega (Madison, WI); C18 reversed-phase HPLC columns (4.6 × 250 mm) from Vydac (Hesperia, CA); cellulose-coated TLC sheets (20 × 20 mm) from EM Science; glutathione-agarose from Sigma; and PhosphorImager screens and scanner from Amersham Biosciences (Piscataway, NJ). Calmodulin was

* This work was supported by United States Public Health Services Grants NS17660 and NS28710 (to M. B. K.) and by a fellowship from the John Douglas French Foundation for Alzheimer Research (to J. S. O.). 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.

‡ To whom correspondence should be addressed. Tel.: 626-395-3923; Fax: 626-395-8474; E-mail: kennedym@its.caltech.edu.

[1] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; CaMKII, calmodulin-dependent protein kinase II; PSD, postsynaptic density; GST, glutathione *S*-transferase; GAP, GTPase-activating protein; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; DIV, days *in vitro*; DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS/MS, tandem mass spectrometry; HCSS, HEPES-control salt solution; ctm, carboxyl-terminal synGAP mutant.

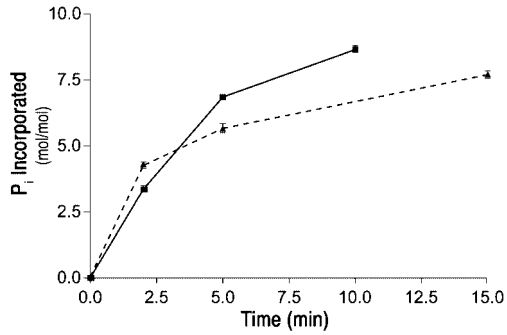

FIG. 1. **Stoichiometry of phosphorylation of synGAP by CaMKII.** Recombinant synGAP (2 μg) in a Hi-5 membrane fraction (*solid line*) was phosphorylated in the presence of exogenous purified CaMKII (3 μg). Native synGAP (2.6 μg) in the PSD fraction (*dashed line*) was phosphorylated with the endogenous CaMKII present in the PSD fraction (total PSD protein, 50 μg). Reactions were carried out in the presence of [γ-^{32}P]ATP (1000 cpm/pmol), Ca^{2+}, and calmodulin, as described under "Experimental Procedures." At the indicated times, reactions were stopped by addition of SDS-PAGE sample buffer. Radiolabeled synGAP was fractionated by SDS-PAGE on 7.5% gels and visualized with a PhosphorImager. [γ-P^{32}]PO_4 in the synGAP protein band was quantified with the use of ImageQuaNT software from Amersham Biosciences, as described under "Experimental Procedures."

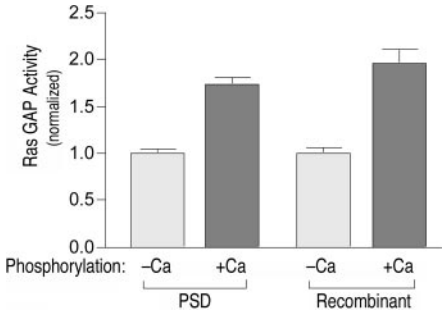

FIG. 2. **Effect of phosphorylation of synGAP by CaMKII on Ras GAP activity.** Ras GAP activity was assayed before and after phosphorylation of synGAP by CaMKII as described under "Experimental Procedures." synGAP in the PSD fraction (15 μg of total PSD protein) was phosphorylated by endogenous CaMKII. Recombinant synGAP (15 μg of Hi-5 cell membrane protein) was phosphorylated by 3 μg of added purified CaMKII. Phosphorylation of synGAP was carried out in the absence ($-Ca$) or presence ($+Ca$) of 0.7 mM $CaCl_2$ and 0.6 μM calmodulin (10 μg/ml) for 2 min at 30 °C. Data are mean values from four independent experiments. GAP activities are plotted after subtraction of intrinsic Ras GTPase activity and are normalized to the activity of nonphosphorylated synGAP. Non-normalized GAP activity, before phosphorylation and after subtraction of the intrinsic Ras GTPase activity, was 11.6% hydrolysis of GTP for synGAP in the PSD fraction and 19% for recombinant synGAP. The mean intrinsic Ras GTPase activity was 6.0% hydrolysis of GTP.

purchased from Calbiochem (San Diego, CA). CaMKII was purified from rat forebrain as previously described (17).

Preparation of Postsynaptic Density Fraction from Rat Brain—The crude PSD fraction was prepared as described previously (6) by a modification of the method of Carlin *et al.* (18).

Expression and Purification of GST Fusion Proteins Containing Portions of synGAP—A vector for expression of a fusion protein containing a portion of the carboxyl tail of synGAP (residues 946–1167) fused to the carboxyl terminus of glutathione *S*-transferase (GST-ctsynGAP) was constructed in the pGEX plasmid, according to the manufacturer's instructions (Amersham Biosciences). The fusion protein contained the sequence of synGAP encoded by positions 2836–3501 in the synGAP cDNA (accession number AF048976). *E. coli* cells transformed with the plasmids were grown in cultures containing 50 μg/ml ampicillin at 37 °C. At mid-log phase, expression was induced by addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside and continued until late log phase. Cells were harvested and frozen at −80 °C. Cells were lysed by sonication in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3 (PBS), containing 1% Triton X-100, proteinase inhibitor mixture (Roche Applied Science, Mannheim, Germany), 0.1 mM PMSF, 0.5 mM dithiothreitol (DTT). After centrifugation at 15,000 × *g* for 10 min, lysate supernatants were incubated at room temperature for 2 h, or at 4 °C overnight with glutathione-agarose beads. In some cases, to recover more fusion protein, the pellets were also resuspended in PBS plus 1% *n*-lauroyl sarcosine, 1% Triton X-100, and 0.1 mM PMSF. The suspension was sonicated and subjected to centrifugation at 15,000 × *g*. The supernatant from this spin was pooled with the lysate supernatants and incubated with glutathione-agarose beads for 2 h at 4 °C. The bead suspension was transferred to a column, and the beads were washed with PBS. GST fusion proteins were eluted in 50 mM Tris (pH 8.0), 20 mM reduced glutathione, and 0.1 mM PMSF at 4 °C. The protein concentration was determined by a modification of the method of Lowry (19) and stored at −80 °C.

Expression of synGAP in Insect Cells—The entire sequence encoding synGAP was inserted into plasmid pVL1392 (BD Pharmingen, San Diego, CA) at EcoR1 and BamH1 restriction sites, which added a FLAG tag to the amino terminus. The recombinant FLAG-tagged synGAP (rsynGAP) was expressed in Hi-5 insect cells by the Caltech Protein Expression Laboratory. Cells were harvested by centrifugation, and the cell pellets were frozen at −80 °C. Pellets were resuspended in 20 mM Tris-Cl (pH 8.0), 2 mM EDTA, 2 mM DTT, 0.1 mM PMSF, 0.5% Triton X-100, 1 μg/ml deoxyribonuclease I, and protease inhibitor mixture (Roche Applied Science) and lysed by homogenization at 4 °C. Nuclei were removed by centrifugation at 100 × *g*, and membranes were harvested at 100,000 × *g*. Like endogenous synGAP, rsynGAP is tightly bound to membranes. All attempts to extract it resulted in loss of GAP activity. Membrane fractions from control insect cells in which synGAP was not present had no detectable GAP activity (data not shown); thus, we are able to reliably measure synGAP activity in the membrane fractions. Recombinant synGAP in the membranes was detected by immunoblotting with an anti-FLAG antibody (Sigma).

Phosphorylation of synGAP and GST-ctsynGAP by CaMKII—Phosphorylation by purified CaMKII was carried out in a reaction mix containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.4 mM EGTA, 30 μM [γ-^{32}P]ATP (1000–3000 cpm/pmol) or 30 μM ATP, 10 μg/ml calmodulin, 10 mM DTT, 3 μg of purified rat brain CaMKII and Hi-5 cell membranes containing 1.5–2 μg of rsynGAP, or 45 ng of purified CaMKII and 3 μg of GST-ctsynGAP. Phosphorylation was initiated by addition of CaMKII and ATP to a 30-μl reaction mix prewarmed to 30 °C for 2 min. The reaction was carried out for 2 min, or as indicated, and stopped by the addition of SDS-PAGE sample buffer. The mixture was placed in a boiling water bath for 3 min then samples were fractionated by SDS-PAGE on 10% gels for GST-ctsynGAP, and 7.5% gels for rsynGAP. The gels were dried and exposed to x-ray film to identify phosphorylated proteins. To quantify the amount of phosphate incorporated, the level of ^{32}P in the bands was determined with a Storm PhosphorImager (Amersham Biosciences). The relative densities measured by the Imager were converted to counts per minute by comparison to signals from standard amounts of [^{32}P]phosphate spotted onto filter paper and imaged at the same time.

Determination of Stoichiometry of Phosphorylation of synGAP by CaMKII—The moles of synGAP per milligram of protein in either the postsynaptic density or the membranes of Hi-5 insect cells was determined from immunoblots containing increasing amounts of protein from each sample, labeled with affinity-purified primary antibodies specific for synGAP (Affinity Bioreagents, Golden, CO), and secondary antibodies conjugated to Alexa fluor-488 (Molecular Probes, Eugene, OR). The labeling was quantified with the use of the Storm system and compared with the labeling of standard amounts of GST-ctsynGAP protein, which contains the epitopes of synGAP used to prepare the primary antibody. We then calculated the nanomoles of phosphate incorporated into the synGAP band (determined as described above) per nanomoles of total synGAP.

Trypsinization of Phosphorylated GST Fusion Proteins and Recombinant synGAP—Proteins were phosphorylated as described above with the following modifications. The reaction was conducted in a volume of 500 μl containing 80–120 μg/ml GST fusion protein or 300–400 μg/ml Hi-5 membrane protein containing recombinant synGAP. The reaction mixture was preincubated at 30 °C for 5 min, and the reaction was initiated by addition of 2–4 μg/ml CaMKII for phosphorylation of GST fusion protein or 10–20 μg/ml CaMKII for phosphorylation of membrane-bound recombinant synGAP in the presence of 30 μM [γ-^{32}P]ATP (1500–3000 cpm/pmol). The reaction was continued for 5, 15, or 30 min at 30 °C and stopped by addition of 3× SDS sample buffer. The mixture was placed in a boiling water bath for 3 min then fractionated by SDS-PAGE. The gel was stained with Coomassie Blue R-250, and the appropriate band of GST fusion protein or recombinant synGAP was identified by comparison to molecular weight markers. Bands were

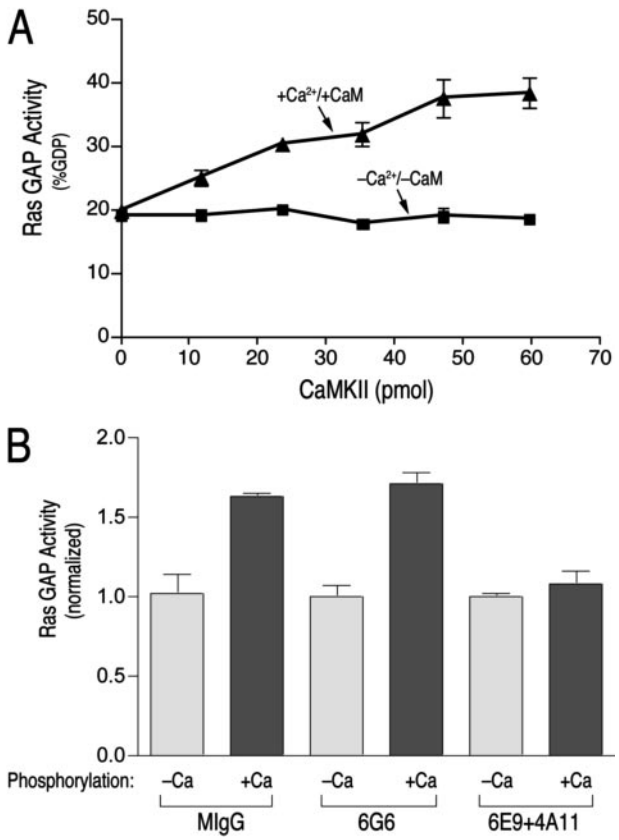

FIG. 3. **Increase in the GAP activity of synGAP depends on CaMKII.** *A*, dependence on amount of CaMKII in the phosphorylation reaction. Recombinant synGAP (15 μg) was phosphorylated for 2 min at 30 °C in the presence of the indicated amounts of purified forebrain CaMKII, then assayed for GAP activity as described under "Experimental Procedures." ■, phosphorylation in the absence of $CaCl_2$ and calmodulin; ▲, phosphorylation in the presence of $CaCl_2$ and calmodulin. *B*, effect of antibodies that inhibit CaMKII on the increase in GAP activity. Monoclonal antibodies 6E9 and 4A11, which inhibit CaMKII activity, were incubated with the PSD fraction (15 μg of each) at 4 °C for 30 min, and then phosphorylation was carried out at 30 °C in the presence of $CaCl_2$ and calmodulin as described above. In two control reactions, either mouse IgG or an anti-PSD-95 antibody (6G6) were substituted for inhibiting antibodies. The data are mean values of three independent experiments. GAP activities are plotted after subtraction of the intrinsic Ras GTPase activity and are normalized to the activity of nonphosphorylated synGAP. Non-normalized GAP activity, before phosphorylation and after subtraction of intrinsic Ras GTPase activity, was 14.5% hydrolysis of GTP in the presence of mouse IgG (MigG), 12.5% in the presence of 6G6, and 15.2% in the presence of 6E9 plus 4A11. The mean intrinsic Ras GTPase activity was 6.8% hydrolysis of GTP.

excised, chopped into small pieces, and transferred to 1.5-ml Eppendorf tubes. The pieces were incubated for 30 min at 37 °C in 2 mM tris-(2-carboxyethyl)phosphine hydrochloride, 50% acetonitrile, 0.5 M ammonium bicarbonate (pH > 8) to reduce the protein and destain the gel piece. The protein was then alkylated by transferring the gel piece to 25 mM iodoacetamide, 50% acetonitrile, 25 mM ammonium bicarbonate and incubating at room temperature in the dark for 20 min. Gel pieces were rinsed with 50 mM ammonium bicarbonate. Proteins were then trypsinized in the gel as previously described (20).

HPLC Fractionation of Phosphopeptides—Trypsinized phosphopeptides were fractionated by HPLC on a C18 reversed-phase column developed at 1 ml/min with a gradient of 0–30% acetonitrile in 0.1% trifluoroacetic acid. Radioactivity in each 0.5-ml fraction was measured in a Beckman LS 7800 scintillation counter by detection of Cerenkov radiation.

Mass Spectrometry and Sequencing of Phosphopeptides—Mass spectrometry was conducted by the Protein and Peptide Microanalytical Laboratory at Caltech with a PerSeptive Biosystems/Vestec Lasertech II reflector for matrix-assisted, laser desorption ionization, time-of-flight (MALDI-TOF) mass spectrometry. Data were collected in both linear and reflector modes. Serine phosphopeptides are reliably identified by appearance of a new peptide in reflector mode that is reduced in mass by ~98 atomic mass units from the parent because of cleavage of a phosphate group from the parent. The identity of some of the phosphopeptides that we detected was confirmed by amino acid sequencing by Tandem mass spectrometry (MS/MS).

Identification of Tryptic Peptides with Peptidesort Software—The Peptidesort software package (GCG, Accyleris, Inc.) permits identification of a peptide from its molecular mass and its relative retention time during HPLC. We used the program to predict all possible tryptic peptides, sorted by retention time and molecular mass, from the amino acid sequence of synGAP. We compared the retention times from HPLC and the molecular masses of each phosphopeptide that we detected by mass spectrometry with those predicted by the program. Mass spectrometry measures the mass of the peptide fragment plus a proton, whereas the program predicts the mass of the corresponding hydrolysis product. Therefore, for comparison, we subtracted 17 atomic mass units from the mass of each peptide predicted by the program. After this correction, unless noted, the differences between masses predicted by the program, and those of peptides determined by mass spectrometry and reported in Tables I and II (see below), were less than 1 atomic mass unit.

Assay of Ras GTPase-activating Activity—GAP assays of 20–35 μg of PSD and 5–15 μg of recombinant synGAP were performed after phosphorylation as described above, in the absence or presence of 0.3 mM free Ca^{2+} and 10 μg/ml calmodulin for 2 min at 30 °C, but in a final volume of 30 μl. In some reactions, antibody 6G6 against PSD-95 or inhibiting antibodies 4A11 and 6E9 (21) against CaMKII (20 μg each of IgG partially purified from Ascites fluid by precipitation with 50% ammonium sulfate) were included in the reaction. The phosphorylation reactions were stopped by addition of 60 μl of a mixture to bring the solution to 0.66 mM EGTA, 11.7 μM okadaic acid, 22 mM Tris-Cl (pH 7.5), 9.3 mM $MgCl_2$, 111 mM NaCl, and 2.2 mM DTT. The GAP assay was then initiated by addition of 10 μl containing 2 pmol of [α-^{32}P]GTP-bound GST-Ras fusion protein (10) for a total volume of 100 μl of GAP reaction mixture (22). The reactions were carried out at 30 °C for 15 min, stopped by addition of 600 μl of ice-cold 5% glutathione-agarose beads and 80 μl of ice-cold 50 mM EDTA, and then incubated at 4 °C for 45 min on an end-over-end mixer. Beads were washed three times (23), nucleotides were dissociated from the column-bound GST-Ras, and [α-^{32}P]GTP and [α-^{32}P]GDP were separated by thin layer chromatography (22). The separated nucleotides were visualized by PhosphorImager analysis (Amersham Biosciences) and quantified with ImageQuaNT software to determine the percent GDP generated in the assay (GDP/[GDP + GTP] × 100), a measure of Ras GTPase activity.

Site-directed Mutagenesis of Recombinant synGAP—We prepared various mutant constructs to study the effect of mutation of the identified phosphorylation sites to alanine (serines 764, 765, 1058, 1062, 1064, 1093, 1095, and 1123). Mutagenic oligonucleotides (18- to 25-mer) that contained alanine instead of the identified serine were synthesized at the Caltech Oligonucleotide Synthesis Laboratory. The oligonucleotides were phosphorylated at the 5′ end by T4 kinase then annealed to the denatured synGAP plasmid (pVL1392) at room temperature for 30 min. The oligonucleotides were extended with T4 DNA polymerase and T4 DNA ligase *in vitro* to generate a hemi-methylated, double-stranded DNA molecule. A restriction digestion was performed with Dpn-1 to eliminate non-mutant plasmid DNA (those with two methylated strands). The DNA molecules were then transformed into the *E. coli* mutS strain (deficient in the methylation-specific repair system), and colonies were screened by DNA sequencing for plasmids containing the alanine mutations (24). Plasmids containing the desired mutations were transformed into *E. coli* DH5α for propagation, and mutations were confirmed by DNA sequencing.

Production of Phosphosite-specific Antibodies—Synthetic peptides with the sequence ITKQH-S(PO_3)-QTPSTC (P-S1123) and RGLNS-S(PO_3)-MDMARC (P-S765) were purchased from SynPep (Dublin, CA). Purified peptides were conjugated via succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate to keyhole limpet hemocyanin. Rabbit antisera against the conjugated P-S1123 and P-S765 were raised by CoCalico Biologicals (Reamstown, PA) and Sigma Genosis (The Woodlands, TX), respectively. Specificity and optimum dilution were determined for each bleed by immunoblotting against 15–20 μg of PSD fraction phosphorylated by endogenous CaMKII, and of equivalent non-phosphorylated PSD fraction. Antibodies from sera that contained a high titer specific for phospho-synGAP were purified by peptide affinity chromatography.

Five milligrams of P-S1123 or P-S765 peptide was conjugated to 5 ml of Sulfolink coupling resin (Pierce, Rockford, IL) according to the manufacturer's instructions. The coupled resin was then mixed for 1 h with

a blocker consisting of 0.1 M cysteine in TE85 (50 mM Tris, pH 8.5, 5 mM EDTA), then washed twice with 5 volumes each of TE85, TE85, 1 M NaCl, and finally with 50 mM Tris, pH 7.5, 5 mM EDTA (TE75). Before each use, the resin was blocked for 1 h with 3 volumes of TE75, 20% non-immune rabbit serum, washed with TE75, G elution buffer (Pierce), and finally re-equilibrated with TE75 buffer. IgG from ~25 ml of serum was concentrated by precipitation in 50% ammonium sulfate, 0.1 M Tris-Cl, pH 7.5. The pellet was redissolved in 50 ml of TE75 and dialyzed against two changes of the same buffer. The dialyzed protein was stirred with the peptide resin for 2 h, poured into a column, and washed with 10 column volumes of TE75. Bound IgG was eluted in Gentle Elution Buffer (Pierce), collecting 1-ml fractions into tubes containing 0.1 ml of 1 M Tris-Cl, pH 7.5. Protein was detected in each fraction by absorbance at 280 nm. Fractions containing high amounts of protein were further characterized by immunoblotting against both phosphorylated and nonphosphorylated PSD protein. Fractions with the highest concentration of phosphosite-specific antibodies were pooled.

Preparation of Cortical Neuronal Cultures—Cultures of cortical neurons with less than 1% astrocytes were prepared from fetal mice (15–16 days gestation) as previously described (25) in 24-well plates coated with 50 ng/ml poly-D-lysine (Sigma) and 2 ng/ml laminin (BD Biosciences) and Neurobasal medium (Invitrogen), B27 supplement (Invitrogen), 0.5 mM Glutamax I (Invitrogen), 25 μM glutamate, and 25 μM β-mercaptoethanol. After 3 days *in vitro* (DIV), cytosine arabinoside (Sigma) was added (10 μM) to halt the growth of non-neuronal cells. Cells were used at 13–14 DIV.

Cell Treatment and Protein Extraction —Cell cultures (13–14 days *in vitro* (DIV)) were washed three times in HEPES-control salt solution (HCSS) containing (in mM): 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 10 NaOH, 20 HEPES, and 5.5 glucose, pH 7.4, and then half were exposed to 25 μM *N*-methyl-D-aspartic acid (NMDA) dissolved in HCSS or to an equal amount of additional HCSS for 15 s (2–4 wells for each condition). After treatment, cells were washed quickly with ice-cold PBS and extracted with lysis buffer (1% SDS, 20 mM Tris-Cl, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 2.5 mM $MgCl_2$, 2 mM orthovanadate, and complete mini protease inhibitor mixture (Roche Applied Science)). Extracts were heated at 90 °C for 5 min, and insoluble material was removed by centrifugation at 14,000 × *g* for 30 min. Protein concentrations were determined by the bicinchoninic acid method (Pierce) using bovine serum albumin as standard.

Western Blotting—To determine synGAP phosphorylation, 5 μg of protein samples was dissolved in SDS-PAGE sample buffer, heated at 90 °C for 5 min, fractionated by SDS-PAGE on 8% gels, and transferred to nitrocellulose membranes (Schleicher & Schuell) in transfer buffer containing 50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol. Membranes were blocked with 5% milk in TBS-T buffer (20 mM Tris, 150 mM NaCl, 0.05% Tween 20) and were then incubated with phosphorylation site-specific antibodies anti-*p*-synGAP-1123 (1:5,000), anti-*p*-synGAP-765 (1:10,000) or antibody recognizing total synGAP (1:3,000). Bound antibodies were detected by the enhanced chemiluminescence method (Pierce).

RESULTS

Stoichiometry and Rate of Phosphorylation of synGAP by CaMKII—synGAP in the postsynaptic density and recombinant synGAP are phosphorylated rapidly and to a high stoichiometry by CaMKII (Fig. 1). We were unable to remove synGAP from the PSD fraction or from Hi-5 cell membranes without destroying its GAP activity; therefore, we performed the experiments with synGAP in the PSD fraction phosphorylated by endogenous CaMKII, and with recombinant synGAP in Hi-5 cell membranes phosphorylated by purified rat forebrain CaMKII, as described under "Experimental Procedures." The most rapid phosphorylation occurs within 2 min after which synGAP contains ~3–3.5 mol of phosphate/mol, suggesting that there are three to four preferred sites of phosphorylation by CaMKII.

The Ras-GTPase Activating Activity of synGAP Is Increased after Phosphorylation by CaMKII—We had previously reported that the Ras GAP activity of synGAP is inhibited about 2-fold after phosphorylation by CaMKII (10). We later found that this observation was caused by inhibition of GAP activity by ATP and pyrophosphate added to inhibit endogenous protein phosphatases (16). We re-investigated regulation of GAP activity of synGAP by CaMKII, after optimizing conditions for the GAP assay in the absence of pyrophosphate, as described under "Experimental Procedures." To preserve the phosphorylation state of synGAP during the GAP assay (15 min), we added EGTA and 3.5 μM okadaic acid at the end of the phosphorylation reaction. These agents fully inhibited dephosphorylation of synGAP and did not interfere with the GAP assay (data not shown). We then redetermined conditions under which the GAP activity is linear with time and with amount of synGAP. Under these conditions, phosphorylation by CaMKII increased the GAP activity of synGAP in the PSD fraction by ~75% and of recombinant synGAP by ~ 95% (Fig. 2).

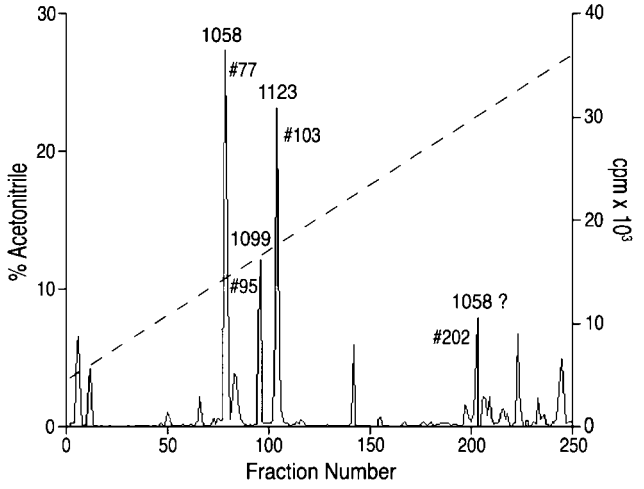

FIG. 4. **Tryptic phosphopeptides generated from the GST-ctsynGAP fusion protein after phosphorylation by CaMKII.** Purified GST-ctsynGAP fusion protein (60 μg), containing residues 946–1167 of synGAP, was phosphorylated for 30 min in the presence of [γ^{32}P]ATP at 30 °C. The phosphorylated protein was fractionated by SDS-PAGE and digested in the gel with trypsin as described under "Experimental Procedures." Tryptic peptides were eluted and fractionated by HPLC, and the indicated radiolabeled tryptic peptides were identified by mass spectrometry as described under "Experimental Procedures" (Table I and Fig. 5). Peaks are labeled with the fraction number (#) and the location of phosphorylated sites in full-length synGAP corresponding to the phosphopeptide in the peak. Fractionation of trypsinized fusion protein after 5 and 15 min of phosphorylation produced peaks with the same retention times. *Dashed line*, acetonitrile concentration.

To verify that phosphorylation by CaMKII is responsible for the increase in activity, we determined the dependence of the increase on the amount of exogenous CaMKII in the phosphorylation reaction (Fig. 3*A*). When no CaMKII was added to the assay, GAP activity was not increased. The importance of phosphorylation by CaMKII for the increase in GAP activity was further supported by an experiment in which we added inhibiting antibodies against CaMKII to the phosphorylation reaction (Fig. 3*B*). The increase in GAP activity in the PSD fraction was decreased when antibodies that inhibit CaMKII (4A11 and 6E9) were included in the phosphorylation reaction but was unaffected by addition of non-immune mouse IgG or an antibody against PSD-95 (6G6).

Identification of Phosphorylation Sites in the Carboxyl-terminal Portion of synGAP—We constructed and expressed various fusion proteins containing portions of synGAP and tested them for phosphorylation by CaMKII as described under "Experimental Procedures." The fusion protein containing the carboxyl-terminal fragment of synGAP (amino acids 946–1167) was rapidly phosphorylated (data not shown), therefore we identified its phosphorylation sites. Tryptic peptides were generated from the phosphorylated protein and fractionated by HPLC as described under "Experimental Procedures." Four major peaks

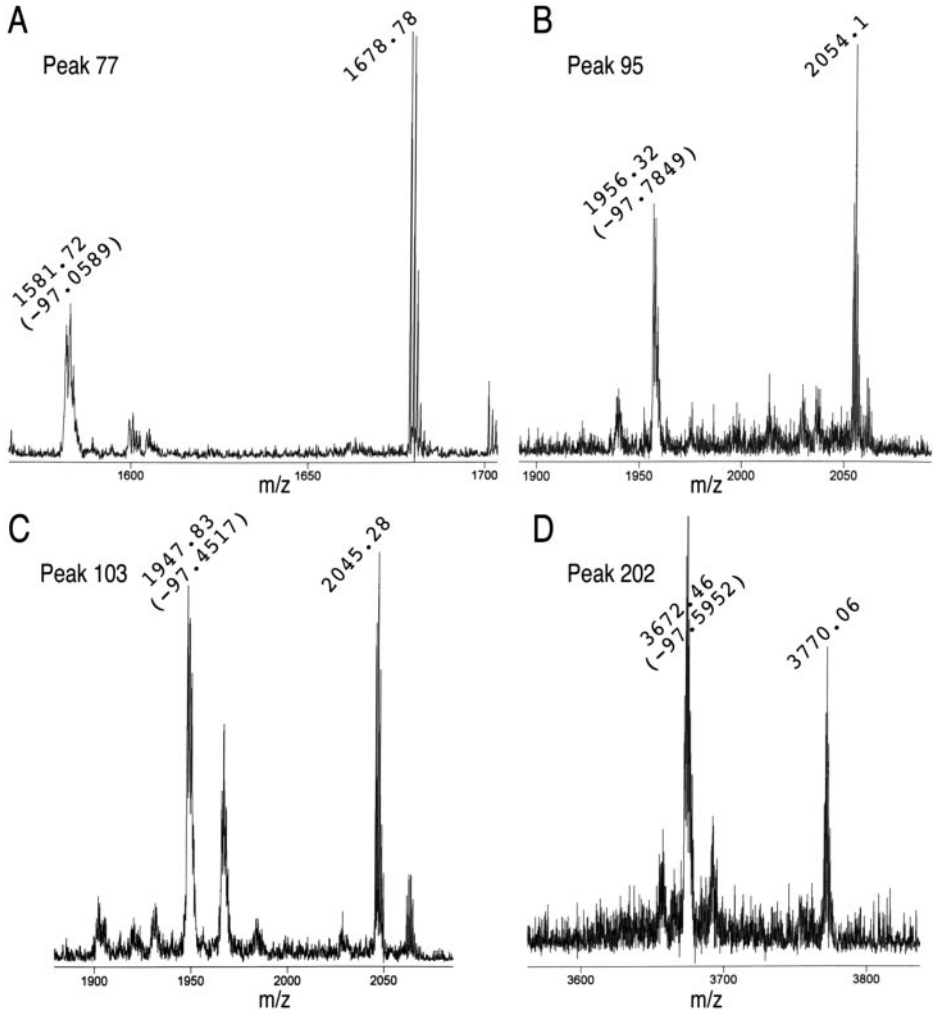

FIG. 5. **Mass spectrographs of phosphopeptides from the GST-ctsynGAP fusion protein.** Postsource decay spectra of phosphopeptide peaks 77 (*A*), 95 (*B*), 103 (*C*), and 202 (*D*) shown in Fig. 4. The *ordinates* are detector counts. Each spectrum shows corrected masses ($M + H^+$) of peptides run in reflector mode in a MALDI-TOF spectrometer. The larger mass is that of the phosphopeptide. The smaller mass is that of the peptide after neutral loss of phosphate (~98).

of radioactivity appeared reproducibly (Fig. 4). The same four peaks were present after 5, 15, or 30 min of phosphorylation, with the size of the peaks proportional to the time of phosphorylation, indicating that all the peaks represent sites that are phosphorylated at approximately the same rate. Individual fractions containing the peaks were concentrated and subjected to MALDI-TOF mass spectrometry in both linear and reflector modes. In reflector mode, neutral loss of H_3PO_4 from serine and threonine phosphopeptides usually produces a new peptide peak with a mass of 98 atomic mass units less than that of the phosphopeptide itself (26). The appearance of this peak is diagnostic for a phosphopeptide. Each of the four samples showed one such new peak in reflector mode (Fig. 5 and Table I), permitting us to identify the mass of the phosphopeptide in each sample. Three of the four phosphopeptides were pure enough to sequence by MS/MS as described under "Experimental Procedures." The sequences revealed that fractions 77, 95, and 103 contained phosphorylated sites corresponding to serines 1058, 1099, and 1123 in full-length synGAP, respectively (Table I). The masses of the predicted tryptic peptides calculated with the program Peptidesort corresponded to those determined by mass spectrometry, confirming the identities of these phosphopeptides. The recovery of the phosphopeptide in fraction 202 was too low for sequencing by MS/MS. Its predicted mass could correspond to one of two peptides: a peptide containing residues 970–1004, which contains 7 serines and 3 threonines, or a peptide containing residues 1056–1090 resulting from partial tryptic digestion of the region containing phosphorylation site serine 1058. The predicted masses of both of these peptides are slightly greater than 1 atomic mass unit different from the mass determined by mass spectrometry (Table I). Thus, the identity of this phosphopeptide remains ambiguous. Its low abundance suggests either that it is a partial digestion product, or that it may not be a preferred phosphorylation site.

Identification of Additional Phosphorylation Sites after Mutation of S1058 and S1123 in Full-length synGAP—To check for additional phosphorylation sites in synGAP, we constructed and expressed full-length synGAP in which the two major phosphorylation sites, S1058 and S1123, were mutated to alanine (S1058/1123A) as described under "Experimental Procedures." This mutant protein was still rapidly phosphorylated by purified CaMKII but to a stoichiometry of only ~1.5 mol of PO_4/mol of synGAP compared with 3–3.5 for wild type synGAP (Fig. 1), indicating that the two mutated sites are indeed major sites phosphorylated rapidly in the native protein. However, the stoichiometry of greater than 1 indicated that additional sites might be present in the portions of synGAP that were not contained in GST-ctsynGAP or might be unmasked in the carboxyl-terminal portion by mutation of the major sites. A tryptic peptide map of the phosphorylated S1058/1123A mutant, prepared as described in the previous section, revealed five major phosphopeptide peaks (Fig. 6). MALDI-TOF mass spectrometry in linear and reflector modes, revealed the mass of phosphopeptides in fractions 79, 88, and 214 (Fig. 7 and Table II). Fractions 98 and 152 contained several peptides, but none of them could be positively identified as a phosphopeptide. We were able to match the masses of phosphopeptides determined

TABLE I
Identification of phosphopeptides in HPLC fractions shown in Fig. 4

The molecular masses of phosphopeptides shown in Fig. 4 were determined by MALDI-TOF mass spectrometry (Fig. 5). The identity of the phosphopeptide in fraction 202 is ambiguous, because two peptides, a partial tryptic product containing site S1058 and a tryptic peptide containing 7 serines and 3 threonines, have masses slightly more than 1 atomic mass unit different from the determined mass.

HPLC fractions	Identified sites	Peptide mass (MALDI-TOF)		Predicted peptide mass[b]	Residues in synGAP
		Linear	Reflected[a]		
		atomic mass units			
77[c]	S1058	1678.78	1581.72	1580.87	1056–1070
95[c]	S1099	2054.10	1956.32	1957.10	1097–1120
103[c]	S1123	2045.28	1947.83	1948.10	1121–1138
202	S1058 or 7 Ss, 3 Ts	3770.06	3672.46	3674.06 or 3673.90	1056–1090 or 970–1004

[a] Mass of peptide after neutral loss of phosphate in reflector mode.
[b] The mass of peptides predicted from tryptic hydrolysis in Peptidesort software were corrected by subtracting 17 atomic mass units to match the $M + H^+$ mass measured by MALDI-TOF.
[c] Identity of phosphopeptide was confirmed by sequencing by MS/MS as described under "Experimental Procedures."

by mass spectrometry and their relative retention times during HPLC with those predicted in the program Peptidesort for specific tryptic peptides (Table II). The phosphopeptide in fraction 79 is predicted to contain two serines at positions 1093 and 1095 in synGAP. The prominent phosphopeptide in fraction 88 is predicted to contain two adjacent serine residues at positions 764 and 765. The phosphopeptide in fraction 214 contains three serines at positions 750, 751, and 756. The two pairs of serines, 750/751 and 764/765, are located in two 13 residue tandem repeats that are 61% identical. These five potential sites between positions 750 and 765 are outside the region of the protein contained in GST-ctsynGAP and represent potential major sites of phosphorylation. In contrast, sites 1093 and 1095 were present in GST-ctsynGAP but were not significantly phosphorylated in the presence of Ser-1058 and Ser-1123 (Fig. 4), indicating that these sites may be minor sites in the wild type protein that are "unmasked" in the mutant. We could not identify the phosphoprotein present in fraction 98, but its retention time is similar to that of the peak containing phosphorylation site 1099 in Fig. 4; thus, Ser-1099 may be significantly phosphorylated in both mutant and wild type synGAP.

The Major Phosphorylation Sites, Serines 764/765, 1058, and 1123, All Contribute to Regulation of GAP Activity of synGAP by CaMKII—To determine which of the identified phosphorylation sites are responsible for regulation of the GAP activity of synGAP, we generated a series of synGAP mutants in which several of the phosphorylation sites, or combinations of them, were mutated to alanine. We then measured the GAP activity after phosphorylation of these mutants by CaMKII as described in Fig. 2. The increase in GAP activity produced by phosphorylation was reduced from ~70% to ~ 40% in a mutant in which both Ser-764 and Ser-765 were changed to alanine (Fig. 8). In contrast, a mutant missing both Ser-1058 and Ser-1123 phosphorylation sites still showed nearly a 70% increase in GAP activity after phosphorylation. Three of the other identified phosphorylation sites, 1093, 1095, and 1099, are located near 1058 and 1123 and are phosphorylated in the mutant that is missing 1058 and 1123. It is possible that phosphorylation, of those sites or of other nearby serines such as 1062 and 1064, can mimic the effect of the phosphorylation of 1058 and 1123. Therefore, we designed a carboxyl-terminal synGAP mutant (ctm) in which six of the potential phosphorylation sites, including 1058, 1062, 1064, 1093, 1095, and 1123, are mutated to alanine. (We have not yet successfully mutated site 1099 to alanine.) The increase in GAP activity induced by phosphorylation of this mutant (termed the ctm mutant) was reduced to about 40%, similar to the increase observed for the 764/765 mutant (Fig. 8). We decided to determine the increase in GAP activity in a mutant in which all eight of these serine to alanine mutations are combined. In this mutant, the increase

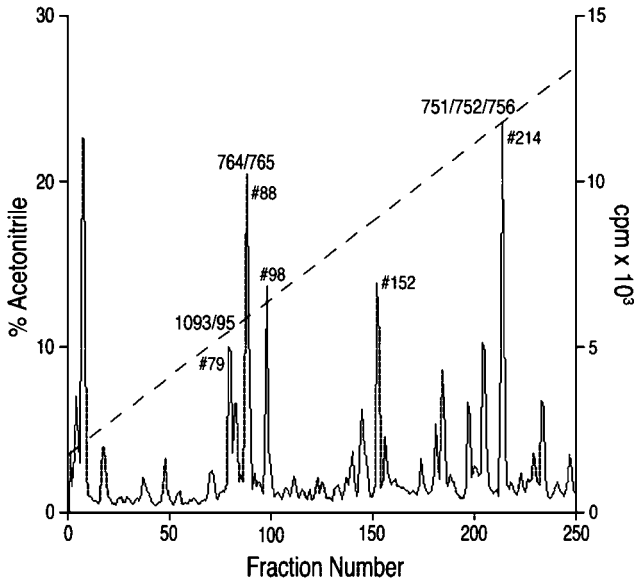

FIG. 6. **Tryptic phosphopeptides generated from recombinant synGAP missing sites S1058 and S1123 after phosphorylation by CaMKII.** A cDNA encoding synGAP with two mutations, S1058A and S1123A (henceforth referred to as S1058/1123A), was expressed in Hi-5 cells as described under "Experimental Procedures." The mutant protein (200 μg) was phosphorylated for 30 min at 30 °C, as described under "Experimental Procedures." The phosphorylated protein was fractionated by SDS-PAGE and digested in the gel with trypsin as described under "Experimental Procedures." Tryptic peptides were eluted and fractionated by HPLC, and the indicated radiolabeled tryptic peptides were identified by mass spectrometry as described under "Experimental Procedures" (Table II and Fig. 7). Peaks are labeled with the fraction number (#) and the location of phosphorylated sites in full-length synGAP corresponding to the phosphopeptide in the peak. Fractions 98 and 152 had detectable radioactivity, however, no phosphopeptides were detected in them by MALDI-TOF mass spectrometry in the reflector mode.

in GAP activity after phosphorylation was reduced to ~20% (Fig. 8). Thus, it appears that these phosphorylation sites all can contribute to the regulation of GAP activity by CaMKII. It is possible that the remaining 20% increase in GAP activity results from phosphorylation of serines 750, 751, 756, or 1099, which we have not yet mutated.

Activation of NMDA Receptors Increases Phosphorylation of synGAP on Ser-1123 and Ser-765 in Cortical Neurons—We raised phosphosite-specific antibodies against phosphorylated peptides containing the sequences surrounding Ser-765 and Ser-1123, as described under "Experimental Procedures." After affinity purification on columns substituted with the peptide antigens, these antibodies are highly specific for phosphorylated synGAP on immunoblots of the PSD fraction (Fig. 9*A*). To

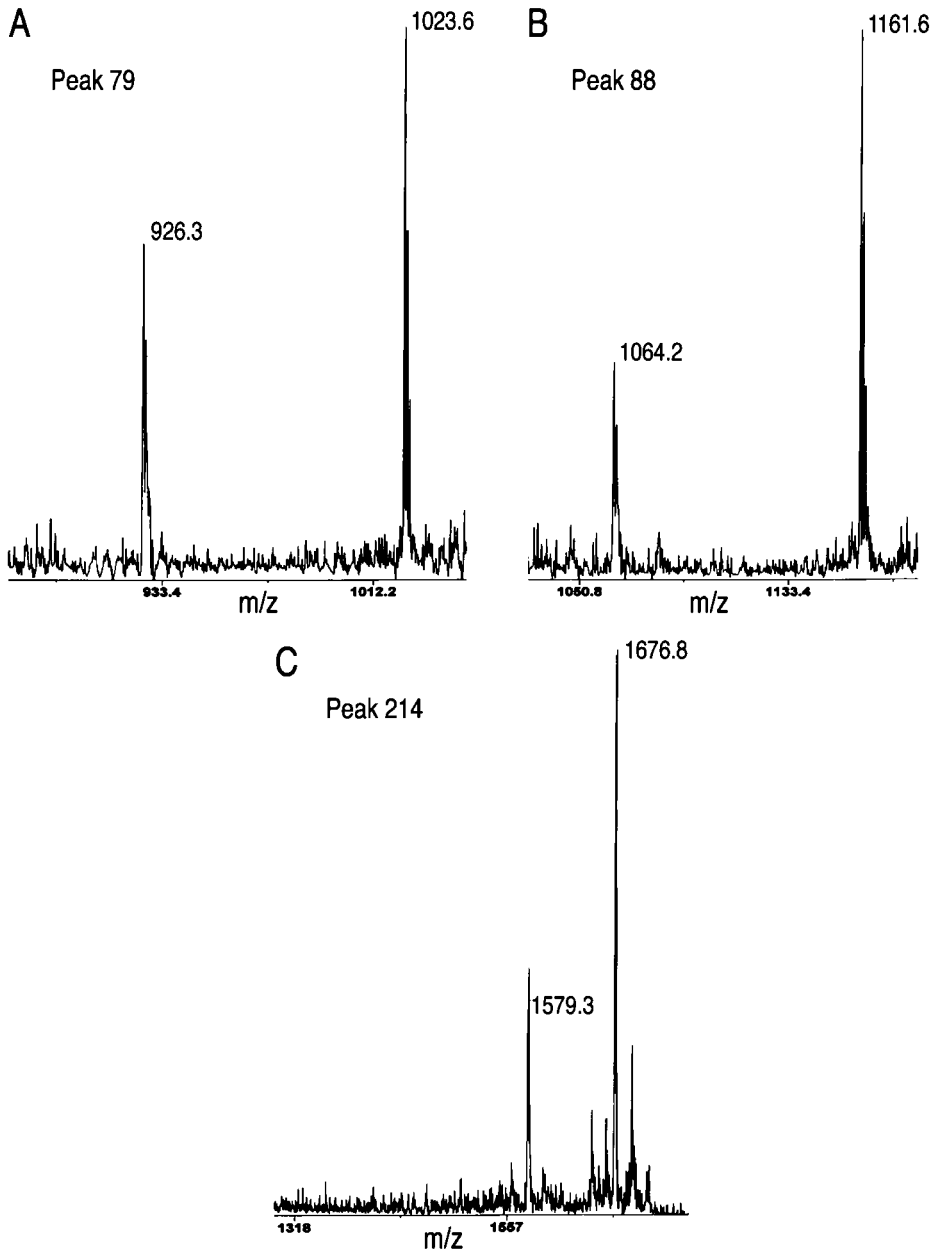

FIG. 7. **Mass spectrographs of phosphopeptides from synGAP mutant missing sites 1058 and 1123.** Post-source decay spectra of phosphopeptide peaks 79 (*A*), 88 (*B*), and 214 (*C*) shown in Fig. 6. The *ordinates* are detector counts. Each spectrum shows corrected masses ($M + H^+$) of peptides run in reflector mode in a MALDI-TOF spectrometer. The larger mass is that of the phosphopeptide. The smaller mass is that of the peptide after neutral loss of phosphate (~98).

determine whether Ser-765 or Ser-1123 can undergo phosphorylation in neurons, cortical cultures were exposed to 25 μM NMDA for 15 s, after which the cultures were extracted with SDS-PAGE sample solution. Immunoblots of extracts from control and treated cultures were prepared and probed with the antibodies against phosphosites and with antibodies against synGAP to control for sample loading. As shown in Fig. 9*B*, treatment of the cultures with NMDA resulted in an increase in phosphorylation of synGAP on both sites, indicating that these sites can be phosphorylated *in vivo* following activation of NMDA receptors.

DISCUSSION

synGAP is an Ras GTPase-activating protein that was originally discovered as an abundant PSD protein that associates with the NMDA-receptor scaffold protein PSD-95 (10, 11). It is a prominent component of a signaling complex that associates with the cytosolic face of the NMDA receptor at postsynaptic spines of excitatory synapses in the central nervous system and regulates a wide variety of synaptic functions in the developing and adult nervous systems (3, 27). Mouse mutants in which synGAP is eliminated by homologous recombination die in the first or second week after birth, indicating that synGAP is essential for viability after birth (14, 15). synGAP is phosphorylated by the Ca^{2+}-regulated protein kinase CaMKII, which is one of the major targets of Ca^{2+} flowing through activated NMDA receptors. To better understand how synGAP may participate in synaptic regulation through the NMDA-receptor, we set out to characterize how synGAP is modified by CaMKII.

We report here that phosphorylation of synGAP by CaMKII increases its GAP activity by 70–95%. The increase in activity is observed both when native synGAP present in the PSD fraction is phosphorylated by CaMKII that is endogenous to the PSD fraction, and when recombinant synGAP in Hi-5 cell membranes is phosphorylated by CaMKII purified from rat forebrain. synGAP is phosphorylated rapidly at several sites in its carboxyl half (Fig. 10), including serines 751/752 and/or 756, serines 764/765, serine 1058, and serine 1123. We have not distinguished between the two sets of adjacent serines, 751/752/756 and 764/765; it seems likely that the adjacent serines may be phosphorylated interchangeably in individual mole-

TABLE II
Identification of phosphopeptides in HPLC fractions shown in Fig. 6

The molecular masses of phosphopeptides shown in Fig. 6 were determined by MALDI-TOF mass spectrometry (Fig. 7). Phosphopeptides in fractions 79, 88, and 214 were identified by comparison with retention order and molecular masses predicted by the Peptidesort program, as described under "Experimental Procedures."

HPLC fractions	Identified sites	Peptide mass (MALDI-TOF)		Predicted peptide mass[b]	Residues in synGAP
		Linear	Reflected[a]		
				atomic mass units	
79	S1093/1095	1023.6	926.3	926.1	1089–1096
88	S764/765	1161.6	1064.2	1064.2	761–770
214	S750/751/756	1676.8	1579.3	1579.7	747–760

[a] Mass of peptide after neutral loss of phosphate in reflector mode.
[b] The mass of peptides predicted from tryptic hydrolysis in Peptidesort software were corrected by subtracting 17 atomic mass units to match the M + H^+ mass measured by MALDI-TOF.

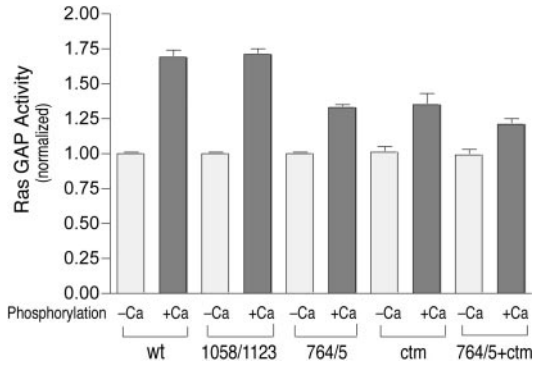

FIG. 8. **Loss of regulation of GAP activity in phosphosite mutants of synGAP.** The indicated synGAP mutants were generated by site-directed mutagenesis and expression in Hi-5 cells as described under "Experimental Procedures." GAP assays were performed for 15 min after phosphorylation by CaMKII in the presence or absence of Ca^{2+}/CaM for 2 min as described under " Experimental Procedures." The data are mean values from four or more independent experiments. GAP activities are plotted after subtraction of intrinsic Ras GTPase activity and are normalized to the activity of non-phosphorylated synGAP. Non-normalized GAP activity, before phosphorylation and after subtraction of Ras GTPase, was 14% hydrolysis of GTP for wt synGAP; 11.8% for S1058/1123A; 14.1% for S764/5A; 17.2% for ctm (S1058A, S1062A, S1064A, S1093A, S1095A, and S1123A); and 17.4% for S764/5A plus ctm. The mean intrinsic Ras GTPase activity was 6.5% hydrolysis of GTP.

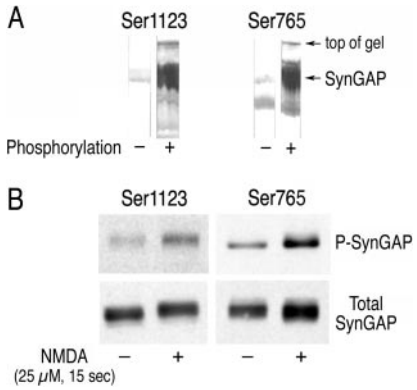

FIG. 9. **Phosphorylation of sites 765 and 1123 is regulated *in vivo* by activation of NMDA-type glutamate receptors.** *A*, antisera against synthetic phosphopeptides with the sequence surrounding serine 765 or serine 1123 in synGAP were generated, and sera with high titers were affinity-purified on columns substituted with the appropriate antigenic peptide, as described under "Experimental Procedures." Fractions were assayed by immunoblotting against purified postsynaptic density proteins before or after phosphorylation by CaMKII, as described under "Experimental Procedures." Fractions with the greatest specificity for phospho-synGAP were pooled. Immunoblots of non-phosphorylated (−) and phosphorylated (+) postsynaptic density proteins were made with 1/20,000 dilutions of the pooled antibodies. The area shown in the figure includes proteins at the top of the gel down to molecular mass ~96 kDa. *Arrows* indicate the top of the gel and the position of synGAP. *B*, cultures of cortical neurons prepared as described under "Experimental Procedures" were exposed to 25 μM *N*-methyl-D-aspartate or to vehicle for 15 s, as described under "Experimental Procedures." Cultures were immediately dissolved in SDS-PAGE sample solution, boiled, fractionated by gel electrophoresis, and immunoblotted with affinity-purified antibodies prepared against phosphopeptides with the sequence surrounding serine 765 or serine 1123 in synGAP, as described under "Experimental Procedures." Similar results were obtained in two experiments.

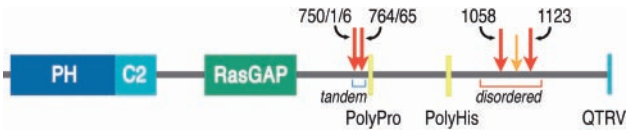

FIG. 10. **Location of phosphorylation sites in synGAP.** The diagram indicates the locations of previously identified domains in the primary sequence of synGAP (10, 11) and the phosphorylation sites identified in this report. The sites indicated by the *large arrows* are identified above them. The *small arrow* shows the location of serines 1093, 1095, and 1099. The *orange bracket* indicates the boundaries of the disordered region predicted by the PONDRs software. The *blue bracket* indicates the boundaries of the tandem repeats containing the phosphorylation sites between residues 750 and 765.

cules. Experiments with phosphosite-specific antibodies that recognize synGAP when it is phosphorylated at serine 765 or at serine 1123 indicate that phosphorylation of both of these sites is increased in cortical neurons within 15 s after activation of NMDA receptors. We have not yet determined whether phosphorylation of these sites in living neurons is catalyzed exclusively by CaMKII. Other sites on synGAP that may also be phosphorylated, but appear from our biochemical results to be less favored, include serines 1093, 1095, and 1099. We generated mutants in which all of the phosphorylated residues, except serines 751, 752, 756, and 1099, were changed to alanine, singly or in combination. Measurement of GAP activity after phosphorylation of the mutants by CaMKII shows that no single site is responsible for the entire increase in activity. Mutation of serines 764/65 or of serines 1058 and 1123 decreases the level of activation by CaMKII. After mutation of six of the identified sites, phosphorylation of CaMKII still increases GAP activity by ~20%. Thus, there are likely additional sites, perhaps serines 751,752, 756, 1099, or sites that we have not yet identified, at which phosphorylation can produce a small increase in GAP activity.

It is interesting that several of the phosphorylation sites that we identified (1058, 1093/95, 1099, and 1123) are located in a region of high disorder spanning residues 1014–1144 (data not shown), as predicted by the software PONDRs (Predictor of Natural Disordered Regions, supplied by A. K. Dunker, Washington State University, Pullman, WA (28–30)). Disordered regions are predicted to be partially or fully unfolded, and, thus, to lack a fixed tertiary structure. PONDRs has been used to detect an association between regions of intrinsic disorder and signaling functions in cell-signaling proteins (31, 32). Such

regions have also been shown to be involved in DNA recognition, modulation of specificity or affinity of protein binding, and protein regulation (33). The two principal phosphorylation sites, 1058 and 1123, are both immediately preceded by a stretch of polyglycine 13 or 14 amino acids long, broken only by a few serine residues, which is predicted to be maximally flexible. We detected phosphorylation of sites 1093/95 only in the S1058A and S1123A double mutant. Thus, their phosphorylation may be due to the structural flexibility of this region, permitting phosphorylation of nearby sites in the absence of the favored sites 1058 and 1123.

We recently found that mutant mice in which synGAP has been deleted either from conception, or conditionally, a few weeks after birth, have abnormally high levels of neuronal apoptosis in several brain regions.[2] In addition, preliminary evidence suggests that neurons cultured from mutant mice have alterations in the timing of activation of Ras following activation of NMDA receptors.[3] Knowledge of the phosphorylated sites that produce increased GAP activity of synGAP, and the availability of antibodies that specifically recognize those phosphorylated sites, will provide tools to unravel the precise roles of synGAP in regulation of synaptic Ras and neuronal apoptosis.

Acknowledgments—We wish to thank Dr. Hong-Jung Chen for some of the synGAP DNA constructs, Alan J. Rosenstein for excellent technical assistance, Dr. A. K. Dunker for help with PONDRs software, and members of the Kennedy laboratory for many helpful discussions.

REFERENCES

1. Sjostrom, P. J., and Nelson, S. B. (2002) *Curr. Opin. Neurobiol.* **12,** 305–314
2. Sheng, M., and Sala, C. (2001) *Annu. Rev. Neurosci.* **24,** 1–29
3. Kennedy, M. B. (2000) *Science* **290,** 750–754
4. Leonard, A. S., Lim, I. A., Hemsworth, D. E., Horne, M. C., and Hell, J. W. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3239–3244
5. Bayer, K. U., De Koninck, P., Leonard, A. S., Hell, J. W., and Schulman, H. (2001) *Nature* **411,** 801–805
6. Cho, K.-O., Hunt, C. A., and Kennedy, M. B. (1992) *Neuron* **9,** 929–942
7. Kornau, H.-C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995) *Science* **269,** 1737–1740
8. Kennedy, M. B. (1997) *Trends Neurosci.* **20,** 264–268
9. Chen, H.-J., and Kennedy, M. B. (1997) *Abstr. Soc. Neurosci.* **23,** 1466 (abstr.)
10. Chen, H.-J., Rojas-Soto, M., Oguni, A., and Kennedy, M. B. (1998) *Neuron* **20,** 895–904
11. Kim, J. H., Liao, D., Lau, L.-F., and Huganir, R. L. (1998) *Neuron* **20,** 683–691
12. Zhang, W., Vazquez, L., Apperson, M. L., and Kennedy, M. B. (1999) *J. Neurosci.* **19,** 96–108
13. McCormick, F., Martin, G. A., Clark, R., Bollag, G., and Polakis, P. (1991) *Cold Spring Harbor Symp. Quant. Biol.* **56,** 237–241
14. Komiyama, N. H., Watabe, A. M., Carlisle, H. J., Porter, K., Charlesworth, P., Monti, J., Strathdee, D. J., O'Carroll, C. M., Martin, S. J., Morris, R. G., O'Dell, T. J., and Grant, S. G. (2002) *J. Neurosci.* **22,** 9721–9732
15. Kim, J. H., Lee, H. K., Takamiya, K., and Huganir, R. L. (2003) *J. Neurosci.* **23,** 1119–1124
16. Oh, J. S., Chen, H.-J., Rojas-Soto, M., Oguni, A., and Kennedy, M. B. (2002) *Neuron* **33,** 151
17. Miller, S. G., and Kennedy, M. B. (1985) *J. Biol. Chem.* **260,** 9039–9046
18. Carlin, R. K., Grab, D. J., Cohen, R. S., and Siekevitz, P. (1980) *J. Cell Biol.* **86,** 831–843
19. Peterson, G. L. (1983) *Methods Enzymol.* **91,** 95–119
20. Hellman, U., Wernstedt, C., Gonez, J., and Heldin, C.-H. (1995) *Analyt. Biochem.* **224,** 451–455
21. Molloy, S. S., and Kennedy, M. B. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4756–4760
22. Bollag, G., and McCormick, F. (1995) *Methods Enzymol.* **255,** 161–170
23. Smith, D. B., and Corcoran, L. M. (1995) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and K., S., eds) Vol. 2, pp. 1671–1677, Wiley Interscience, New York
24. Kramer, B., Kramer, W., and Fritz, H. J. (1984) *Cell* **38,** 879–887
25. Rose, K., Goldberg, M., and Choi, D. W. (1993) in *In Vitro Biological Methods. Methods in Toxicology* (Tyson, C., and Frazier, J., eds) pp. 46–60, Academic Press, San Diego
26. Annan, R. S., and Carr, S. A. (1996) *Anal. Chem.* **68,** 3413–3421
27. Sheng, M., and Pak, D. T. (2000) *Annu. Rev. Physiol.* **62,** 755–778
28. Li, X., Obradovic, Z., Brown, C. J., Garner, E. C., and Dunker, A. K. (2000) *Genome Inform. Ser. Workshop Genome Inform.* **11,** 172–184
29. Romero, P., Obradovic, Z., Li, X., Garner, E. C., Brown, C. J., and Dunker, A. K. (2001) *Proteins* **42,** 38–48
30. Li, X., Romero, P., Rani, M., Dunker, A. K., and Obradovic, Z. (1999) *Genome Inform. Ser. Workshop Genome Inform.* **10,** 30–40
31. Iakoucheva, L. M., Brown, C. J., Lawson, J. D., Obradovic, Z., and Dunker, A. K. (2002) *J. Mol. Biol.* **323,** 573–584
32. Dunker, A. K., Brown, C. J., Lawson, J. D., Iakoucheva, L. M., and Obradovic, Z. (2002) *Biochemistry* **41,** 6573–6582
33. Dyson, H. J., and Wright, P. E. (2002) *Curr. Opin. Struct. Biol.* **12,** 54–60

[2] I. Knuesel and M. B. Kennedy, unpublished data.
[3] P. Manzerra and M. B. Kennedy, unpublished data.