WD40 Repeat Proteins Striatin and S/G2 Nuclear Autoantigen Are Members of a Novel Family of Calmodulin-binding Proteins That Associate with Protein Phosphatase 2A*

(Received for publication, June 17, 1999, and in revised form, December 23, 1999)

Carlos S. Moreno‡, Susan Park§§, Kasey Nelson‡, Danita Ashby‡, Frantisek Hubalek‡, William S. Lane§, and David C. Pallas‡‡**

From the ‡Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322, the §§Division of Cellular and Molecular Biology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115, and the ¶¶Harvard Microchemistry Facility, Harvard University, Cambridge, Massachusetts 02138

Protein phosphatase 2A (PP2A) is a multifunctional serine/threonine protein phosphatase that is critical to many cellular processes including development, neuronal signaling, cell cycle regulation, and viral transformation. PP2A has been implicated in Ca2+-dependent signaling pathways, but how PP2A is targeted to these pathways is not understood. We have identified two calmodulin (CaM)-binding proteins that form stable complexes with the PP2A A/C heterodimer and may represent a novel family of PP2A B-type subunits. These two proteins, striatin and S/G2 nuclear autoantigen (SG2NA), are highly related WD40 repeat proteins of previously unknown function and distinct subcellular localizations. Striatin has been reported to associate with the post-synaptic densities of neurons, whereas SG2NA has been reported to be a nuclear protein expressed primarily during the S and G2 phases of the cell cycle. We show that SG2NA, like striatin, binds to CaM in a Ca2+-dependent manner. In addition to CaM and PP2A, several unidentified proteins stably associate with the striatin-PP2A and SG2NA-PP2A complexes. Thus, one mechanism of targeting and organizing PP2A with components of Ca2+-dependent signaling pathways may be through the molecular scaffolding proteins striatin and SG2NA.

PP2A, a serine/threonine protein phosphatase found in all eukaryotic cells, regulates a wide variety of important cellular events, including DNA replication, transcription, translation, development, neuronal signaling and progression of the cell cycle (for reviews see Refs. 1–3). The PP2A heterotrimer consists of a catalytic (C) subunit, a structural (A) subunit, and a regulatory (B-type) subunit (4). Although relatively few C- and A-type subunits have been identified, multiple B-type subunits exist, including B (or B55), B’ (or B56), and B’’ (or PR72/130) classes (5–9). To enable utilization of this phosphatase for numerous substrates in different pathways, PP2A is regulated at multiple levels, including covalent modifications, interactions with inhibitory proteins and lipids, and association with the various B-type subunits. For example, B’ subunits were recently shown to target PP2A to the adenomatous polyposis coli tumor suppressor scaffolding protein, physically associating PP2A with specific substrates and thus regulating Wnt-β-catenin signaling (10).

PP2A has also been shown to form complexes with CaM-dependent kinase IV (CaMKIV) (11), suggesting a role for PP2A in Ca2+-dependent signaling. This possibility is further supported by patch clamp experiments with both neuronal (12) and smooth muscle cells (13) that have used both okadaic acid and recombinant PP2A C subunit to implicate PP2A in the regulation of calcium-activated potassium channels and L-type Ca2+ channels (14).

To better understand how PP2A is targeted to various microenvironments and signal transduction pathways within the cell, we have looked for additional PP2A targeting subunits. Here we report the identification of two PP2A-associated proteins that may represent a novel family of B-type subunits. These two proteins contain WD40 repeats and bind to CaM in a calcium-dependent manner. One member of this family, striatin, is localized to the post-synaptic densities of neuronal dendrites (15), whereas the other, SG2NA, has been reported to be localized to the nucleus (16). Striatin-PP2A and SG2NA-PP2A complexes contain several additional unidentified proteins, suggesting that striatin and SG2NA may function as scaffolding proteins involved in Ca2+-dependent signal transduction pathways.

EXPERIMENTAL PROCEDURES

Metabolic Labeling and Immunoprecipitations—For metabolic labeling of NIH3T3 cells with methionine, subconfluent dishes of cells were labeled for 4–6 h with 0.25 μCi/ml [35S]methionine in methionine-free Dulbecco’s modified Eagle’s medium supplemented with dialyzed 0.5% fetal calf serum. Cells were washed twice with phosphate-buffered saline and once with IP wash buffer (0.135 M NaCl, 1% glycerol, 20 mM Tris, pH 8.0) and then were lysed in 1 ml of IP lysis buffer (0.135 M NaCl, 1% glycerol, 20 mM Tris, pH 8.0, 0.03 units/ml aprotinin, and 1–2 mM phenylmethylsulfonyl fluoride) while rocking for 20 min at 4 °C. Lysates were cleared at 13,000 × g for 1 min at 4 °C and then incubated at 4 °C for 90 min while rocking in 1.5-ml Eppendorf tubes with protein A-Sepharose and the appropriate antisera. Immune complexes were precipitated by centrifugation for 1 min at 700 × g for 20 min at 4 °C. Lysates were cleared at 13,000 × g for 1 min at 4 °C and then incubated at 4 °C for 90 min while rocking in 1.5-ml Eppendorf tubes with protein A-Sepharose and the appropriate antisera. Immune complexes were precipitated by centrifugation for 1 min at 700 × g, and the supernatants were removed. Immune complexes were washed twice with 1 ml of IP lysis buffer and three times with 1 ml of phosphate-buffered saline. Two-dimensional gel electrophoresis was performed as described previously (17), and proteins were transferred to nitrocellu-
lose membranes. Autoradiographs were performed with Biomax MS Film (Kodak).

Preparative Immunopurification—Using affinity-purified AR-1 anti-
s sera, samples were immunopurified from 40 15-cm dishes of polyoma-
virus MT-transformed NIH3T3 cells as described (17) except that the IP
lysis buffer contained 20% Triton and 1% glycerol. For preparative
immunoprecipitations, PP2A complexes were immunospecifically purified
using the 16d anti-C subunit monoclonal antibody (mAb) chemically
cross-linked to protein A-Sepharose as described previously (18) except
that 10 15-cm dishes of cells were used for one batch purification and
whole immune complexes were analyzed on two-dimensional (2D) gels
(17). Control immunopurifications were performed with 7-34-1 mAb
coupled to protein A-Sepharose using five 15-cm dishes of
cells. Proteins were visualized with Coomassie Brilliant Blue R250
(Bio-Rad).

Ion Trap Mass Spectroscopy—Specific spots at 93 and 110 kDa were
subjected to in-gel reduction, carboxymethylation, and tryptic
digestion (Promega). Multiple peptide sequences were determined in
a single run by microcapillary reverse-phase chromatography directly
connected to a Finnigan LCQ quadrupole ion trap mass spectrometer. The
ion trap was programmed to acquire successive sets of three scan modes
consisting of full scan MS over alternating ranges of 395–800 m/z or
800–1300 m/z, followed by two data-dependent scans on the most abun-
dant ion in those full scans. These data-dependent scans allowed: 1) the
automatic acquisition of a high resolution (zoom) scan to determine charge
states and exact mass and 2) MS/MS spectra for peptide sequence
information. MS/MS spectra were acquired with a relative collision
energy of 30%, an isolation width of 2.5 daltons, and dynamic exclusion of
ions from repeat analysis. Interpretation of the resulting MS/MS spectra of
the peptides was facilitated by programs developed in the Harvard Microchemistry Facility and by data base correlation with the
algorithm SEQUEST (19, 20).

Antibodies—Lasergene DNASTAR Protein software was utilized to
identify highly hydrophilic and antigenic sequences for selection of
peptide antigens. Rabbit polyclonal pan-B’ (AR-1), striatin, and SG2NA
antiseras were generated using keyhole limpet hemocyanin (KLH)-con-
jugated peptides as immunogens. Peptide DP47 (ELFDSEDPRERD-
FLKTC) corresponds to residues 194–209 of the human α-isof orm of B’
(8567) with an additional carboxyl-terminal cysteine for coupling to
KLH. Peptide DP52 (GESPKQKGQEIKRSSGDC) corresponds to resi-
dues 227–243 of SG2NA with a carboxyl-terminal cysteine for coupling.

Peptide DP53 (SAGVSQPSPRSSFLPES) corresponds to residues 373–
387 of striatin with an added carboxyl-terminal cysteine for coupling.
Peptides were conjugated to KLH using the Imject maleimide KLH
conjugation kit (Pierce) according to the manufacturer’s instructions.
The methylation-sensitive PP2A C subunit mAb, 4b7, was generated to
conjugation kit (Pierce) according to the manufacturer’s instructions.
Peptides were conjugated to KLH using the Imject maleimide KLH
conjugation kit (Pierce) according to the manufacturer’s instructions.

Using affinity-purified AR-1 antiserum, proteins as striatin and SG2NA.

Peptides used for immunoaffinity purification of PP2A complexes were
immunoaffinity purified using the 16d anti-C subunit monoclonal antibody (mAb) chemically
cross-linked to protein A-Sepharose as described previously (18) except
that 10 15-cm dishes of cells were used for one batch purification and
whole immune complexes were analyzed on two-dimensional (2D) gels
(17). Control immunopurifications were performed with 7-34-1 mAb
coupled to protein A-Sepharose using five 15-cm dishes of
cells. Proteins were visualized with Coomassie Brilliant Blue R250
(Bio-Rad).

Ion Trap Mass Spectroscopy—Specific spots at 93 and 110 kDa were
subjected to in-gel reduction, carboxymethylation, and tryptic
digestion (Promega). Multiple peptide sequences were determined in
a single run by microcapillary reverse-phase chromatography directly
connected to a Finnigan LCQ quadrupole ion trap mass spectrometer. The
ion trap was programmed to acquire successive sets of three scan modes
consisting of full scan MS over alternating ranges of 395–800 m/z or
800–1300 m/z, followed by two data-dependent scans on the most abun-
dant ion in those full scans. These data-dependent scans allowed: 1) the
automatic acquisition of a high resolution (zoom) scan to determine charge
states and exact mass and 2) MS/MS spectra for peptide sequence
information. MS/MS spectra were acquired with a relative collision
energy of 30%, an isolation width of 2.5 daltons, and dynamic exclusion of
ions from repeat analysis. Interpretation of the resulting MS/MS spectra of
the peptides was facilitated by programs developed in the Harvard Microchemistry Facility and by data base correlation with the
algorithm SEQUEST (19, 20).

Antibodies—Lasergene DNASTAR Protein software was utilized to
identify highly hydrophilic and antigenic sequences for selection of
peptide antigens. Rabbit polyclonal pan-B’ (AR-1), striatin, and SG2NA
antiseras were generated using keyhole limpet hemocyanin (KLH)-con-
jugated peptides as immunogens. Peptide DP47 (ELFDSEDPRERD-
FLKTC) corresponds to residues 194–209 of the human α-isof orm of B’
(8567) with an additional carboxyl-terminal cysteine for coupling to
KLH. Peptide DP52 (GESPKQKGQEIKRSSGDC) corresponds to resi-
dues 227–243 of SG2NA with a carboxyl-terminal cysteine for coupling.
Peptide DP53 (SAGVSQPSPRSSFLPES) corresponds to residues 373–
387 of striatin with an added carboxyl-terminal cysteine for coupling.
Peptides were conjugated to KLH using the Imject maleimide KLH
conjugation kit (Pierce) according to the manufacturer’s instructions.
The methylation-sensitive PP2A C subunit mAb, 4b7, was generated to
conjugation kit (Pierce) according to the manufacturer’s instructions.
Peptides were conjugated to KLH using the Imject maleimide KLH
conjugation kit (Pierce) according to the manufacturer’s instructions.
RESULTS AND DISCUSSION

Identification of 110- and 93-kDa PP2A-associated Proteins as Striatin and SG2NA—In an effort to discover previously unknown members of the B* family of PP2A subunits, a pan-B* subunit antibody termed AR-1 was generated against a sequence highly conserved in all known B* subunits (Figs. 1A and 2A). AR-1 recognized known B* subunits that migrated at 56 and 74 kDa, as well as three unknown proteins that migrated at 93, 110, and 131 kDa (Fig. 1A). In a parallel attempt to identify PP2A-associated proteins, PP2A immunoprecipitations were prepared from [35S]methionine-labeled NIH3T3 cells using a monoclonal antibody (1d6) directed against the carboxyl terminus of the PP2A C subunit. Several specific spots were observed on 2D gels, including two at 93 and 110 kDa (Fig. 1B). To determine whether the 93- and 110-kDa proteins in 1d6 immunoprecipitates were the same proteins detected in AR-1 immunoblots of whole cell lysates (Fig. 1A), 1d6 immunoprecipitates were immunoblotted with AR-1 (Fig. 1C, lanes 1 and 2). The two proteins that migrated at 93 and 110 kDa were specifically recognized by the AR-1 antisera, suggesting that they might be novel B*-type subunits.

To identify the 110-kDa protein, a large scale direct immunoprecipitation using the AR-1 antisera was subjected to 2D gel electrophoresis, and peptide sequences were obtained by ion trap mass spectrometry (Fig. 1D). These sequences corresponded to a previously cloned gene of unknown function, striatin, which was originally purified from rat brain (15). Striatin has been reported to bind CaM with a 40 nM Kd in a calcium-dependent manner at a half-maximal Ca2+ concentration of 0.5 μM (23). Moreover, striatin contains two polybasic domains that may facilitate association with the post-synaptic membrane (15). Immunolabeling has shown that striatin is excluded from neuronal axons but is found throughout dendrites and is abundant in the post-synaptic densities of neuronal dendritic spines (15). These data suggest that striatin targets PP2A to a cellular microenvironment in which it may play a role in the modulation of calcium-dependent neuronal signaling. Although striatin was originally described as a brain-specific protein, we have observed striatin protein in murine NIH3T3 fibroblasts and human Jurkat T lymphocytes. We have also detected striatin mRNA in human HeLa cervical cancer cells, and expressed sequence tags (ESTs) were found in the dBEST database that represent partial striatin cDNAs from human B lymphocytes, human heart, murine myotubules, and murine testis (data not shown). Thus, striatin is present in both dividing and non-dividing cells and is much more widely expressed than previously thought.

To identify the 93-kDa protein and obtain further peptide sequence from the 110-kDa protein, large scale PP2A (1d6) immunoprecipitations were subjected to 2D gel electrophoresis. Mass spectrometric sequencing revealed multiple additional peptides from striatin for the 110-kDa protein and identified the 93-kDa protein to be a highly related protein, S/G2 nuclear autoantigen (SG2NA) (Fig. 1D). Little is known about SG2NA other than that it is localized to the nucleus, it contains WD40 repeats, and as assayed by immunofluorescence, its expression appears to be cell cycle-regulated, peaking during the S and G2 phases (16). Striatin and SG2NA bear little homology to the B, B* or B** subunits, raising the possibility that they might comprise a new family of PP2A B-type subunits. At least one homolog of striatin and SG2NA exists in Caenorhabditis elegans (GenBankTM accession no. CAAG4873) (24), suggesting that this form of PP2A may play an important role in all metazoans. Although there is no obvious homology for either of these proteins in yeast, potential WD40-containing open reading frames of unknown function with some homology to striatin and SG2NA do exist in both Saccharomyces cerevisiae, (GenBankTM accession no. CAAS9144) and Schizosaccharomyces pombe (GenBankTM accession no. CA21906).

Striatin and SG2NA Share a Conserved Epitope with B* Subunits—Because striatin and SG2NA showed no obvious homology to PP2A B*-type subunits, it was puzzling that they were recognized efficiently by the pan-B* antisemur, AR-1. However, a careful comparison revealed that, whereas neither striatin nor SG2NA contains the precise consensus sequence used to generate the AR-1 antisera, they both contain consensus sequences that share some homology with this motif at positions corresponding to striatin 277–294 and 551–566 (Fig. 2B). To determine whether the AR-1 antisera recognized either or both of these sequences, two peptides were synthesized corresponding to these two sequences as well as two control peptides corresponding to randomized sequences containing the same amino acids. A dot blot (Fig. 2C) demonstrated that the AR-1 antisera recognizes the STR277 peptide corresponding to striatin 277–294 but not the striatin 551–566 peptide or the control peptides. The STR277 peptide was also tested to determine whether it could block immunoprecipitations with the AR-1 antisera (Fig. 2D). As expected, both the STR277 peptide and the AR-1 pep-
tide used to generate the AR-1 antisera were effective at blocking immunoprecipitation of both striatin and SG2NA, whereas the randomized control peptide did not block AR-1 immunoprecipitations. The conservation of the sequence (D/E)(X)(D/S)/T/X/D/E/X16,R/K/E/X19,F/Y)LXT between most B’ subunits and striatin and SG2NA suggests that it may be involved in interactions between these proteins and the A/C heterodimer. Consistent with this hypothesis, AR-1 was able to immunoprecipitate striatin and SG2NA only in the presence of 20% Triton. Under these conditions, little or none of the PP2A A and C subunits are co-immunoprecipitated (data not shown), suggesting that 20% Triton may have dissociated striatin and SG2NA from the A/C heterodimer, revealing the AR-1-specific epitope.

To facilitate the investigation of striatin and SG2NA interactions with the A/C heterodimer, polyclonal antisera were raised against both proteins. The striatin antiserum was raised against a peptide antigen corresponding to amino acids 373–387 of human striatin. This sequence is located within a basic domain that may be important for striatin association with the cellular membrane (15). Although this region is 100% identical between mouse and human striatin, it is not found in SG2NA (Fig. 1D). The SG2NA antiserum was raised against a peptide antigen corresponding to amino acids 227–243 of human SG2NA in a region that has little homology with striatin sequences. 1d6 immunoprecipitations of PP2A were probed with the anti-striatin and anti-SG2NA antiserum, confirming that these antisera recognize the 110- and 93-kDa proteins, respectively (Fig. 1C).

PP2A A and C Subunits Coinmuneimmunoprecipitate with Striatin and SG2NA—To confirm that striatin and SG2NA form stable complexes with PP2A, striatin and SG2NA complexes were immunoprecipitated with the anti-striatin and anti-SG2NA antisera, respectively, analyzed by SDS-PAGE, and immunoblotted with a commercially obtained mAb to PP2A C subunit. Third, immunoblots of striatin and SG2NA immune complexes againstcdc2 phosphorylation sites. The high level of cdc2 phosphorylase a as a substrate, and sensitivity to the PP2A inhibitor okadaic acid was measured. Immunoprecipitations using antisera to striatin, SG2NA, and C subunit (4e1) all contained significant phosphatase activity (Fig. 3B). The measured activity was largely inhibited by 2 mM okadaic acid, as expected for PP2A. These assays were performed in the presence of 1 mM EGTA, indicating that calcium Csubunit is not required for the activity of striatin-PP2A and SG2NA-PP2A complexes. The addition of 2 mM calcium to these phosphatase reactions had a variable but slightly inhibitory effect on PP2A activity (data not shown).

Striatin and SG2NA Activate A/C Heterodimers toward cdc2-phosphorylated Histone H1—Different B-type subunits have been shown to differentially activate the PP2A A/C heterodimer toward different substrates. For example, B subunit is the only known B-type subunit reported to activate A/C heterodimers toward cdc2-phosphorylated histone H1 substrate (50–100-fold) (25–27). To determine whether the presence of striatin and SG2NA in PP2A complexes modulates the activity of A/C heterodimers, radiolabeled cdc2-phosphorylated histone H1 substrate was used to compare the activity of striatin and SG2NA complexes with other forms of PP2A. Immunoprecipitations were prepared from NIH3T3 cells that stably express HA-tagged C subunit (36wt cells) using two different mAbs to the carboxyl terminus of C subunit (1d6 and 4e1) and one mAb to the amino-terminal epitope tag (12CA5). Approximately 10–30% of 12CA5 complexes prepared from 36wt cells contain B subunit, whereas approximately 5% of 16d complexes contain striatin and SG2NA (data not shown); 4e1 complexes contain C subunit alone and A/C heterodimers (18). Striatin and SG2NA complexes immunoprecipitated with anti-striatin and anti-SG2NA antisera, respectively, were highly activated toward histone H1 substrate compared with PP2A in 4e1 and 1d6 immune complexes (Fig. 3C). PP2A immunoprecipitated by 12CA5 had similar (within 2-fold) activity to striatin and SG2NA complexes. Based on our estimate of the percentage of 12CA5 complexes containing B subunit, we would estimate that striatin and SG2NA activate A/C heterodimers less than B subunit toward histone H1. Similar experiments using NIH3T3 cells stably expressing HA-tagged B subunit confirmed this hypothesis (Fig. 3D), with striatin and SG2NA complexes approximately 45 and 29% as active, respectively, as B subunit complexes against cdc2 phosphorylation sites. The high level of observed histone H1 phosphatase activity suggests that striatin and SG2NA are not bound to the A/C heterodimers via the catalytic site.

Chemiluminescence quantitation of immunoblots containing both lysates and immunoprecipitations indicated that approximately 0.1–0.5% of the total C subunit present in lysates was immunoprecipitated with the striatin and SG2NA antisera (data not shown). This finding contrasts with the observation that 5% of 16d immune precipitates contain striatin and SG2NA. Potential explanations for this difference are: 1) not all of the striatin and SG2NA present in lysates was immunoprecipitated; 2) the striatin and SG2NA antisera may partially destabilize complex formation with the A/C heterodimer; or 3) 1d6 may bind preferentially to striatin/PP2A and SG2NA/PP2A complexes.

Multiple Additional Proteins Are Present in Striatin and
protein and used to measure phosphatase activity. Phosphorylase α was immunoprecipitated with 4e1 anti-PP2A C subunit, striatin, and SG2NA antisera. PP2A complexes were simultaneously probed with a mixture of monoclonal antibodies to C subunit (Transduction Laboratories) and A subunit (4 g7 mAb; Ref. 17). These lanes were from the same gel but were not all originally adjacent and have been cut vertically. Although the top and bottom portions of the blot are not shown, no other bands were visible. Whether PP2A C subunit migrates as a doublet or as a single band varies from gel to gel. This behavior on SDS-PAGE has been observed previously (22) and does not appear to be the result of degradation. B, normalized phosphorylase α phosphatase activity of immune complexes prepared with 4e1 anti-PP2A C subunit (PP2A), striatin, and SG2NA antisera. Immunoprecipitates were prepared from approximately 3 mg of total protein and used to measure phosphatase activity. Phosphorylase α phosphatase assays were carried out using the Protein Phosphatase Assay System (Life Technologies, Inc.) according to the manufacturer’s instructions with the addition of 1 mM EGTA and the indicated concentrations of okadaic acid. Cpm released was first corrected by subtracting background activity obtained with pre-immune immunoprecipitations. Preimmune Cpm averaged 7 ± 5% of PP2A activity, 34 ± 13% of SG2NA activity, and 45 ± 15% of striatin activity. The average immune-specific activity measured in the absence of okadaic acid was arbitrarily set to 100% for each immunoprecipitate. The effect of okadaic acid was measured by normalizing Cpm released in the presence of okadaic acid against Cpm released in the absence of okadaic acid. The averages and standard deviations of at least three independent experiments are shown. C, normalized histone H1 phosphatase activity of immunoprecipitations prepared from NIH3T3 cells stably expressing HA-tagged PP2A C subunit (HA-C subunit). Immunoprecipitations were performed using 12CA5 anti-HA-tag mAb; 1d6 and 4e1 anti-C subunit mAbs; and polyclonal antisera against striatin and SG2NA. The averages and standard deviations of at least three independent experiments are shown. Two-thirds of each immunoprecipitate prepared from approximately 3 mg of total protein was used to measure phosphatase activity as described previously (22), and one-third was analyzed by SDS-PAGE and immunoblotted with commercial anti-C subunit mAbs (Transduction Laboratories). The amount of C subunit present in immunoblots of each complex was quantitated using a chemiluminescence imager (Bio-Rad). Cpm released was first corrected by subtracting background activity obtained with pre-immune immunoprecipitations. Specific activity (immune-specific Cpm released/chemiluminescence counts) was then calculated, and the level of phosphatase specific activity was finally normalized relative to the amount of HA-B subunit (HA-B subunit). Immunoprecipitations were performed using 12CA5 anti-HA-tag mAb and striatin and SG2NA polyclonal antisera as described in C. The averages and standard deviations of at least three independent experiments are shown. Calculations were performed as described in C, except specific activity was computed as a percent of HA-tagged PP2A B subunit specific activity.

SG2NA Complexes—The observation that striatin and SG2NA bind to both CaM and PP2A and contain WD40 repeats suggested that these proteins might function as molecular scaffolds for PP2A signaling complexes. To test whether striatin and SG2NA interact with additional proteins, NIH3T3 cells were metabolically labeled with [35S]methionine, and whole cell lysates were immunoprecipitated with both the anti-striatin and anti-SG2NA antisera. Immunoprecipitated complexes were subjected to 2D gel electrophoresis and A subunit, C subunit, striatin, and SG2NA were detected in striatin and SG2NA immunoprecipitations, but not in pre-immune controls (Fig. 4B and data not shown). ImmunobLOTS of SG2NA immunoprecipitates with affinity-purified SG2NA antiserum revealed multiple additional immune-specific bands, suggesting that other members of the SG2NA family may exist (Fig. 4B). The observation that striatin and SG2NA bind to both CaM and PP2A and contain WD40 repeats suggested that these proteins might function as molecular scaffolds for PP2A signaling complexes. To test whether striatin and SG2NA interact with additional proteins, NIH3T3 cells were metabolically labeled with [35S]methionine, and whole cell lysates were immunoprecipitated with both the anti-striatin and anti-SG2NA antisera. Immunoprecipitated complexes were subjected to 2D gel electrophoresis and A subunit, C subunit, striatin, and SG2NA were detected in striatin and SG2NA immunoprecipitations, but not in pre-immune controls (Fig. 4B and data not shown). ImmunobLOTS of SG2NA immunoprecipitates with affinity-purified SG2NA antiserum revealed multiple additional immune-specific bands, suggesting that other members of the SG2NA family may exist (Fig. 4B). The Carboxyl Terminus of PP2A C Subunit Present in Striatin-PP2A and SG2NA-PP2A Complexes Is Highly Methylation—We have previously suggested that the methylation state of the C subunit might regulate the association of A/C heterodimers with B-type subunits (22). To determine the methylation state of the C subunit in striatin-PP2A and SG2NA-PP2A complexes, a portion of the C subunit in striatin and SG2NA immunoprecipitations was subjected to demethylation by base treatment. Both untreated and demethylated samples were analyzed by immunoblot with a methylation-sensitive probe.
mAb (Fig. 5A) that recognizes only the demethylated C subunit. The level of C subunit detected by the methylation-sensitive antibody was substantially enhanced in base-treated samples relative to untreated controls, and chemiluminescence quantitation determined that more than 90% of the C subunits associated with striatin and SG2NA are methylated. This result indicates that C subunit methylation does not prevent striatin and SG2NA association with the A/C heterodimer, but it does not indicate whether methylation is needed for formation of striatin-PP2A and SG2NA-PP2A complexes.

Deletion of the Carboxyl Terminus of PP2A C Subunit Does Not Prevent Striatin-PP2A and SG2NA-PP2A Complex Formation—Although the C subunit carboxyl terminus is essential for formation of PP2A heterotrimers containing the cellular B subunit, it is not required for formation of heterotrimers containing the viral B-type subunit, polyoma virus middle tumor antigen (MT) (22). To investigate the importance of the C subunit carboxyl terminus for the formation of A/C/striatin and A/C/SG2NA heterotrimers, striatin and SG2NA immunoprecipitations were performed from cell lines expressing HA-tagged C subunit mutants (22). The final nine amino acids that contain sites of both phosphorylation (Tyr-307) (28) and methylation (Leu-309) (29) are deleted in one of these mutants (301Stop). The 301Stop mutant was co-immunoprecipitated with striatin and SG2NA at least as efficiently as the wt C subunit (Fig. 5B). These data indicate that striatin and SG2NA interact with the core A/C heterodimer in a fundamentally different manner than the B subunit, behaving more like polyoma virus MT than B subunit in their binding to the A/C heterodimer. Thus, the association of striatin and SG2NA with the A/C heterodimer is probably not directly affected by the covalent modification of the C subunit carboxyl terminus. However, the association of striatin and SG2NA with the A/C heterodimer could be indirectly affected by modifications that influence competition for the A/C heterodimer by altering the affinity of other B-type subunits. Consistent with this possibility, the C subunit mutant, T304A, which has been shown to have an increased affinity for B subunits (22), was reduced 5-fold in its ability to form complexes with striatin and SG2NA compared with the wild-type HA-tagged C subunit (36wt, Fig. 5C).

Taken together, the following observations all strongly suggest that striatin and SG2NA represent a novel family of B-type subunits: 1) striatin and SG2NA complex with the PP2A A/C heterodimer; 2) these complexes contain okadaic acid-sensitive, PP2A-like phosphatase activity; 3) striatin and SG2NA share a conserved epitope with B′ subunits; 4) no known B-type subunits can be observed in these complexes; 5) unlike B subunits, the association of striatin and SG2NA with the A/C heterodimer is independent of the C subunit carboxyl terminus, yet they can activate the A/C heterodimer toward cdc-2 phosphorylated histone H1; and 6) their relative binding to wt and T304A C subunits is opposite that of B subunit (implying that B subunit may even compete with them for binding to the T304A mutant form of the A/C heterodimer). However, we cannot exclude the formal possibility that some other undiscovered B-type subunit that is not recognized by any of the B, B′, or B″ antisera could be present in these complexes. Our conclusion that striatin and SG2NA may be members of a new class of B-type subunits would be further strengthened by additional evidence of competition with other B-type subunits or demonstration of direct interaction between bacterially expressed recombinant striatin and SG2NA and recombinant PP2A A subunit or A/C heterodimer. Should further evidence conclusively demonstrate that striatin and SG2NA are members of a novel family of B-type subunits, we propose that they be designated the B″′ (or B93/110) family.

The finding that striatin and SG2NA form stable complexes with PP2A and might represent a novel (B″′) family of PP2A subunits is the first description of a function for these highly related WD40 repeat proteins. The fact that striatin is highly abundant in post-synaptic membranes, whereas SG2NA appears to be targeted to the nucleus, provides yet another mechanism for the localization of PP2A to different cellular micro-environments. The observation that SG2NA, as well as striatin, binds to CaM in a calcium-dependent manner indicates that these proteins probably link PP2A to calcium-dependent signaling pathways and cellular events. Although
PP2A complexes with CaMKIV have been detected by cross-linking (11), it is not yet known whether this interaction is direct or requires a molecular scaffold. Although we were not able to detect CaMKII or CaMKIV in immunoblots of striatin or SG2NA immunoprecipitations, we have detected kinase activity in these immunoprecipitates (data not shown). Furthermore, the large number of stably associated proteins observed in SG2NA immunoprecipitations suggests that striatin and SG2NA function as molecular scaffolds for the interactions of PP2A with large signal transduction complexes. The identification of additional cellular components of these complexes will provide new insights into the cellular function of striatin, SG2NA, and PP2A.

Acknowledgments—The authors thank Brian Lang, Michael Sanford, and Russell Thomas for technical assistance; Renee Robinson, Dan Kirby, and Kerry Pierce of the Harvard Microchemistry Facility for their expertise in high pressure liquid chromatography and mass spectrometry; and Dr. Brian Hemmings for the gift of anti-E' (PR72/130) polyclonal antisera. Under agreements between Upstate Biotechnology Inc. and Emory University and Calbiochem and Emory University, David Pallas is entitled to a share of sales royalty received by the University from these companies. In addition, this same author serves as a consultant to Upstate Biotechnology Inc. The terms of this arrangement have been reviewed and approved by Emory University in accordance with its conflict of interest policies.

REFERENCES
1. Mumby, M. C., and Walter, G. (1993) Physiol. Rev. 73, 673–699
2. Hopkin, K. (1995) J. NIH Res. 7, 27–30
3. Goldberg, Y. (1999) Biochem. Pharmacol. 57, 321–328
4. Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508
5. Tanabe, O., Nagase, T., Murakami, T., Nozaki, H., Usui, H., Nishito, Y., Hayashi, H., Kagamiyama, H., and Takeda, M. (1996) FEBS Lett. 379, 107–111
6. Zolnierowicz, S., Csortos, C., Bondor, J., Verin, A., Mumby, M. C., and DePaoli-Roach, A. A. (1994) Biochemistry 33, 11858–11867
7. McCright, B., and Virshup, D. M. (1995) J. Biol. Chem. 270, 26123–26128
8. Tehrani, M. A., Mumby, M. C., and Kamibayashi, C. (1996) J. Biol. Chem. 271, 5164–5170
9. McCright, B., Rivers, A. M., Audlin, S., and Virshup, D. M. (1996) J. Biol. Chem. 271, 22081–22089
10. Seeling, J. M., Miller, J. R., Gil, R., Moon, R. T., White, R., and Virshup, D. M. (1999) Science 283, 2089–2091
11. Westphal, R. S., Anderson, K. A., Means, A. R., and Wadzinski, B. E. (1998) Science 280, 1258–1261
12. Tian, L., Knuts, H. G., and Shipston, M. J. (1998) J. Biol. Chem. 273, 13531–13536
13. Zhou, X. B., Ruth, P., Schlossmann, J., Hofmann, F., and Korth, M. (1996) J. Biol. Chem. 271, 19760–19767
14. Groschere, K., Schuhmann, K., Missakes, G., Baumgartner, W., and Romanin, C. (1996) Biochem. J. 318, 513–517
15. Castets, F., Bartoli, M., Bargier, J. V., Baillat, G., Salin, P., Moir, A., Bourgeois, J. P., Demetz, F., Rougon, G., Calothy, G., and Monneron, A. (1998) J. Cell Biol. 134, 1051–1062
16. Muro, Y., Chan, E. K., Landberg, G., and Tan, E. M. (1995) Biochem. Biophys. Res. Commun. 207, 1029–1037
17. Pallas, D. C., Shahrik, L. K., Martin, B. L., Jaspers, S., Miller, T. B., Brautigan, D. L., and Roberts, T. M. (1999) Cell 100, 167–176
18. Ogris, E., Du, X., Nelson, K. C., Mak, E. K., Yu, X. X., Lane, W. S., and Pallas, D. C. (1999) J. Biol. Chem. 274, 14382–14391
19. Chittum, H. S., Lane, W. S., Carlson, B. A., Roller, P. P., Lung, F. D., Lee, R. B., and Hatfield, D. L. (1998) Biochemistry 37, 10866–10870
20. Eng, J. K., McCormick, A. L., and Yates, J. R., III. (1994) J. Am. Soc. Mass Spectrom. 5, 976–989
21. Turo, W., Fernandez, A., Favre, B., Lamb, N. J., and Hemmings, B. A. (1995) J. Cell Biol. 129, 397–410
22. Ogris, E., Gibson, D. M., and Pallas, D. C. (1997) Oncogene 15, 911–917
23. Bartoli, M., Monneron, A., and Sudan, G. (1998) J. Biol. Chem. 273, 22248–22253
24. Wilson R, Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., Coulson, A., Craxton, M., Dear, S., Du, Z., and Durbin, R. (1994) Nature 368, 32–38
25. Ferrigno, P., Laganiere, T. A., and Cohen, P. (1993) Mol. Biol. Cell 4, 669–677
26. Sola, M. M., Laganiere, T., and Cohen, P. (1991) Biochim. Biophys. Acta 1089, 211–216
27. Agostinis, P., Durant, H., Sarno, S., Goris, J., and Merlevede, W. (1992) J. Biochem. 205, 241–248
28. Chen, J., Martin, B. L., and Brautigan, D. L. (1992) Science 257, 1261–1264
29. Lee, J., and Stock, J. (1993) J. Biol. Chem. 268, 19192–19195
WD40 Repeat Proteins Striatin and S/G2 Nuclear Autoantigen Are Members of a Novel Family of Calmodulin-binding Proteins That Associate with Protein Phosphatase 2A
Carlos S. Moreno, Susan Park, Kasey Nelson, Danita Ashby, Frantisek Hubalek, William S. Lane and David C. Pallas

J. Biol. Chem. 2000, 275:5257-5263.
doi: 10.1074/jbc.275.8.5257

Access the most updated version of this article at http://www.jbc.org/content/275/8/5257

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

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

This article cites 29 references, 15 of which can be accessed free at http://www.jbc.org/content/275/8/5257.full.html#ref-list-1