Zinedin, SG2NA, and Striatin Are Calmodulin-binding, WD Repeat Proteins Principally Expressed in the Brain*

Received for publication, December 10, 1999, and in revised form, March 17, 2000
Published, JBC Papers in Press, March 21, 2000, DOI 10.1074/jbc.M909782199

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Striatin is an intracellular protein characterized by four protein-protein interaction domains, a caveolin-binding motif, a coiled-coil structure, a calmodulin-binding domain, and a WD repeat domain, suggesting that it is a signaling or a scaffold protein. Down-regulation of striatin, which is expressed in a few subsets of neurons, impairs the growth of dendrites as well as rat locomotor activity (Bartoli, M., Ternaux, J. P., Forni, C., Portalier, P., Salin, P., Alamric, M., and Monneron, A. (1999) J. Neurobiol. 40, 234–243). Zinedin, a “novel” protein described here, and SG2NA share with striatin identical protein-protein interaction domains and the same overall domain structure. A phylogenetic analysis supports the hypothesis that they constitute a multigenic family deriving from an ancestral gene. DNA probes and antibodies raised against specific domains of each protein showed that zinedin is mainly expressed in the central nervous system, whereas SG2NA, of more widespread occurrence, is mainly expressed in the brain and muscle. All three proteins are both cytosolic and membrane-bound. All three bind calmodulin in the presence of Ca\textsuperscript{2+}. In rat brain, SG2NA and striatin are generally not found in the same neurons. Both localize to the soma and dendrites, suggesting that they share a similar type of addressing and closely related functions.

Striatin is an intracellular protein mostly present in neurons of mammalian basal ganglia and cranial and spinal motor nuclei (1, 2). Electron microscopy showed that it is present in the somato-dendritic compartment of neurons, especially in dendritic spines (1). Brain fractionation shows that striatin is both cytosolic and associated with membranes. This multimeric protein possesses, from the N to the C terminus, four domains mediating protein-protein interactions: a caveolin-binding domain (aa 1–55–63) (3), a putative coiled-coil structure (aa 70–116), a Ca\textsuperscript{2+}-calmodulin (CaM)-binding domain (aa 149–166) (4), and a WD repeat domain (aa 419–780). The WD repeat family is composed of homologous, structurally related, but functionally diverse proteins able to organize multiple simultaneous or consecutive protein-protein interactions (5). The richness of striatin in domains mediating protein-protein interactions suggests that striatin is both a signaling protein and a multimodal platform protein. A study aimed at elucidating the function of striatin revealed two sets of data demonstrating its central role both in embryonic neurons and in adult brain (6). On the one hand, we showed that the expression of striatin is essential for the maintenance and growth of dendrites in rat embryonic motoneurons in culture. On the other hand, we showed that striatin is involved in the control of motor function in adult rats. Albeit a quantitatively minor protein, striatin thus appears to play major cellular and physiological roles.

We have previously reported that the sequence of SG2NA, a 713 aa, supposedly nuclear protein discovered by Muro et al. (7), is 80% similar to and 66% identical to sequences of striatin (1). The expression pattern and domain organization of SG2NA were, however, not studied. The purpose of this work was to characterize SG2NA and to search for proteins homologous to striatin and SG2NA. Thanks to EST data bases, we found a novel protein, zinedin, that shares with striatin and SG2NA identical protein-protein interaction domains. The molecular cloning, chromosomal localization, biochemical properties, and tissue-specific expression of both zinedin and SG2NA are reported here. All three proteins bind CaM in the presence of Ca\textsuperscript{2+}, suggesting that they may play a role in or depend upon Ca\textsuperscript{2+} signaling. Within neurons, striatin and SG2NA are specifically addressed to dendrites. Because homologous proteins were found in Caenorhabditis elegans, fruit fly, Xenopus, and zebra fish, we could perform a phylogenetic analysis leading to the proposal that striatin, zinedin, and SG2NA are proteins deriving from an ancestral gene.

Very recently, Moreno et al. (8) hypothesized that striatin and SG2NA may target the catalytic subunit C of protein phosphatase 2A (PP2A) to components of Ca\textsuperscript{2+}-dependent signaling pathways. Their interesting suggestion is based upon the fact that striatin, SG2NA, and PP2A subunit C were found to coimmunoprecipitate within detergent complexes from lysed NIH-3T3 cells. However, a direct interaction between the C subunit of PP2A and striatin or SG2NA has not been demonstrated.

PP2A, protein phosphatase A2; kb, kilobase(s); nt, nucleotide(s); RT, reverse transcription; PCR, polymerase chain reaction; GST, glutathione S-transferase; TBS, Tris-buffered saline; EST, expressed sequence tag.

* This work was supported by the CNRS, by the Association pour la Recherche sur le Cancer Grants 9318 and 9832, and the Association Française contre les Myopathies Grant FRN 210/6481. 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.

** Supported by a fellowship from the Association pour la Recherche sur le Cancer.

† The abbreviations used are: aa, amino acid(s); CaM, calmodulin;
**Fractionation of Mouse and Rat Brains; Multitissue Western Blots—**

Frozen tissues (heart, skeletal muscle, brain, cerebellum, kidney, lung, liver, and spleen) from 3-month-old mice were homogenized in TBS containing 1 mM EDTA and inhibitors of proteases using a polytron (4). The various homogenates were centrifuged at 150,000 g for 40 min at 4°C. Supernatants and their protein contents were determined (micro BCA method, Pierce). Rat brain fractionation was achieved as described (4). Normalized amounts (100 µg) of protein from each fraction were analyzed in triplicate on 8% SDS-polyacrylamide gels and transferred onto nitrocellulose. The blots were stained with Ponceau red. Striatin, zinedin, and SG2NA were revealed by their respective antibodies using affinity-purified anti-peptide antibodies obtained from their respective antisera. Blots were then blocked using a 1:10,000 dilution of anti-striatin antibodies. The blots were incubated in a 1:20,000 dilution of affinity-purified anti-zinedin antibodies and a 1:125,000 dilution of affinity-purified anti-SG2NA antibodies, washed, and incubated in a 1:20,000 dilution of anti-rabbit antibodies (Vector Laboratories, 1/200), washed, incubated for 2 h in phosphate-buffered saline containing biotinylated streptavidin (Vector). Chromosomes were counterstained and R-banded as described (10). 50 human metaphase cells were analyzed.

**RESULTS—**

Molecular Cloning and Chromosomal Localization of Human Zinedin—The full-length human striatin sequence was used for a TBLASTN search (16) of the NCBI EST database. A number of matching ESTs were found. Their analysis allowed the identification of a hitherto unknown protein, both in mouse and human, homologous to striatin and SG2NA. We determined the sequence of the deduced human cDNA, which encodes a...
protein of 753 aa, called zinedin (Fig. 1). The ATG codon lies within a canonical eukaryotic translation start sequence (17). The 3'-noncoding sequence is terminated by a poly(A) stretch of 34 base pairs. The protein sequence of zinedin encompasses four stretches homologous to those that in striatin encode domains involved in protein-protein interactions (Fig. 2). They are, from the N to the C terminus: a putative caveolin-binding domain (aa 71–79, 89% identical to striatin), a putative coiled-coil structure (aa 86–132, 94% identical), a putative CaM-binding domain (aa 165–182, 79% identical), and a WD repeat domain (aa 395–753, 61% identical). These domains are distributed in the same way as they are in striatin.

The chromosomal position of the human zinedin gene was determined by fluorescent in situ hybridization. In 85% of the metaphases, a fluorescent signal was detected in the q13.2 band on the long arm of chromosome 19 (Fig. 3).

Mapping of SG2N, the Gene Encoding SG2NA, on Chromosome 14 and Existence of Alternate Splicing—As already mentioned, the human protein sequence of SG2NA is 80% homologous to the sequence of striatin (1). These homologies concern all the protein-protein interaction domains that have been described in striatin, from the N to the C terminus: a putative caveolin-binding domain (aa 71–79, 89% identical to striatin), a putative coiled-coil structure (aa 86–132, 94% identical), a putative CaM-binding domain (aa 165–182, 79% identical), and a WD repeat domain (aa 395–753, 61% identical). These domains are distributed in the same way as they are in striatin.

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sequence of SG2NA is devoid of exons 8 and 9. To more precisely map SG2N on chromosome 14, we realized the in situ hybridization of metaphase cells. SG2N lies in the 14q13-q21 region (not shown).

Analysis of the Striatin Family—Proteins homologous to striatin, zinedin, and SG2NA are present in metazoan throughout their evolution; they have been detected in nonmammalian vertebrates (Danio rerio and Xenopus laevis) and in nonvertebrates (Drosophila melanogaster and C. elegans) but not in yeast (18). A phylogenetic analysis supports the hypothesis that the present members of the striatin family derive from an ancestral gene (Fig. 4). Altogether, they are thus entitled to define a family of multimodular proteins, which we propose to name the striatin family. Furthermore, the structures of the human SG2N gene and of the available part of the human STRN gene (clone NH0288C18, Accession no. AC007382) are identical, with the same intron-exon borders. Finally, we have localized the human genes encoding striatin, SG2NA, and zinedin to chromosomes 2p21-p22 (18), 14q13-q21, and 19q13.2. We found that the human genes of two multigenic families, the Na$^+$-Ca$^{2+}$ exchanger family (NCX1, NCX2, and NCX3 localized to chromosomes 2p21-p23, 19q13.2, and 14q21-q31, respectively) (19), and the CaM family (CALM1, CALM2, and CALM3 localized to chromosomes 14q24-q31, 2p21.1-p21.3, and 19q13.2-q13.3, respectively) (20) are in close proximity to the striatin, SG2NA, and zinedin gene loci.

Tissular Expression of Zinedin, SG2NA, and Striatin—Transcripts of the genes encoding striatin, zinedin, and SG2NA were identified in normalized mouse multissue Northern blot triplicates (Fig. 5). As already described (1), two mouse striatin transcripts of 8 and 6 kb were mostly detected in brain and in cerebellum. One 4-kb mouse zinedin transcript was detected in the brain and cerebellum. One 4.5-kb mouse SG2NA transcript was mostly expressed in the brain, cerebellum, and skeletal muscle. Upon longer exposure of the blots, weak signals were detected with all probes in various tissues such as spleen, skeletal muscle and lung for striatin, kidney for zinedin, spleen, kidney and lung for SG2NA.

A human Northern blot was also analyzed with probes corresponding to human sequences (not shown). We found little correlation between the data obtained with the mouse and human Northern blots; for instance, the SG2NA probe gave practically no signal in brain. However, the normalization of the human RNAs was questionable (absence or low level of the actin transcript in several human tissues that express actin). We therefore tend to think that the results obtained with the mouse Northern blots are more reliable.

Next, the proteins themselves, striatin, zinedin, and SG2NA were searched for in several mouse tissues by Western blotting (Fig. 6A). The blots were probed with affinity-purified antibodies specific for each protein. Indeed, no cross-reaction of any of the antibodies had been detected on blots of the three fusion proteins (not shown). As shown previously, an antibody raised against an unconserved striatin peptide revealed a 114-kDa protein, principally in the brain and cerebellum. Very weak signals were detected in the lung and spleen. Anti-zinedin antibodies detected a 100-kDa protein present in the brain, cerebellum, and lung; this protein was very weakly detected in
the heart, kidney, and spleen. Anti-SG2NA antibodies produced signals in almost all tissues. The signals were strongest in brain, cerebellum, muscle, and lung. Using two different sets of anti-SG2NA antibodies, three proteins of 112, 102, and 94 kDa were detected in the brain and cerebellum. In the heart, skeletal muscle, and lung, the antibodies only detected the 94-kDa species. In kidney, an additional protein of 90 kDa was weakly detected. To be sure that the 112-kDa species of SG2NA and striatin were indeed different proteins, a mixture of the three antibodies was used in Western blotting; the two proteins did not comigrate (Fig. 6B, lane 4). Transduction Laboratories developed a monoclonal antibody against striatin (S 66020) with the antigen being a peptide spanning aa 450–600. In Fig. 6B, lane 5, it can be seen that this antibody recognizes both striatin and zinedin.

The major tissular localizations of SG2NA given by the Western blot are in good agreement with that obtained from the Northern blot, except for lung. In the case of zinedin, the level of protein expression in lung is larger than expected from the Northern blot. On the whole, all three proteins are mostly expressed in brain and in the case of SG2NA, in muscle.

Striatin migrates in SDS-polyacrylamide gels with an anomalous apparent $M_r$, 114,000 (theoretical $M_r$, 96,225) (1). Likewise, SG2NA and zinedin display anomalous electrophoretic behaviors migrating at apparent $M_r$, 112,000, 102,000, and 94,000 for SG2NA and 100,000 for zinedin (theoretical $M_r$, 80,592) (Fig. 6).

Brain striatin is found both in the cytosol and in the membrane fraction. In brain homogenates, 58% of striatin (4) and 50–52% of zinedin and SG2NA are membrane-bound and released by detergents (data not shown).

*Zinedin and the Three Species of SG2NA Bind CaM in the Presence of Ca$^{2+}$*—We previously showed that striatin (native and recombinant) binds CaM in a Ca$^{2+}$-dependent manner and that its CaM-binding domain spans aa 149–166 (4). Sequences similar to the latter are found in zinedin and SG2NA (Fig. 7A). To test the ability of zinedin and of the three species of SG2NA to bind CaM *in vitro*, rat brain cytosol was incubated with CaM-Sepharose in the presence of 1 mM Ca$^{2+}$. As shown in Fig. 7B, zinedin quantitatively bound CaM-Sepharose and was eluted in the presence of EDTA. Moreno et al. (8) have shown that the 94-kDa species of SG2NA binds CaM-Sepharose in the presence of Ca$^{2+}$. Fig. 7B shows that the two other species of SG2NA detected in brain also bound CaM and were eluted by EDTA.

**Immunocytochemical Localization of SG2NA in Rat Brain and Adrenal Gland**—Affinity-purified anti-SG2NA antibodies were used. Control sections (using either the primary antibodies preincubated with the immobilized antigen or antibodies from unimmunized animals) were unstained (Fig. 8D). Whatever the tissue (brain or adrenal gland), the cytoplasm, not the nuclei, were immunostained (Fig. 8). In brain sections, many types of brain cells, neurons as well as glial cells, were positive. In all neurons, SG2NA immunoreactivity was present in dendrites but not in axons. In the cerebellum, the soma and dendrites of the Purkinje cells, down to their distal branches, were strongly stained (Fig. 8, A and B). In the granular layer, only the Golgi cells were immureactive (Fig. 8A). The large aspiny interneurons of striatum were intensely immunostained, whereas the spiny, efferent neurons were negative (Fig. 8C). Hippocampal pyramidal and granular cells were strongly reactive (not shown). A few nuclei, such as the trigeminal and the cochlear nuclei, were intensely stained. In the white matter,
astrocytes were immunostained in tracts that do not contain neurons. We also studied SG2NA immunoreactivity in the adrenal gland, a tissue of neural origin. The medulla was strongly reactive, whereas the cortex was not (Fig. 8B).

As shown in Western blots, zinedin is mostly expressed in brain. However, although the anti-zinedin antibodies were able to immunoprecipitate brain native zinedin, they could not be used for immunocytochemistry. Aldehyde fixation of the brain, even using as low a paraformaldehyde concentration as 0.5%, resulted in the absence of zinedin immunoreactivity, most probably because the epitope was drastically modified by the fixative.

**DISCUSSION**

The present study describes two proteins, zinedin and SG2NA, closely related to striatin. Their protein sequences are 66% similar. The three proteins roughly comprise the same number of amino acids and possess four identical protein-protein interaction domains. By contrast, the sequences of the N-terminal regions and of the central regions comprised between the CaM-binding domains and the WD repeat domains bear no homology, a fact that allowed us to raise antibodies specific for each protein. The overall domain structure of these proteins is reminiscent of that of β subunits of heterotrimeric G proteins, because the latter also possess, in the same order, a coiled-coil structure, a CaM-binding domain (according to Liu et al. (21)), and a WD repeat domain.

The striatin sequence 70–116 is identified by computer algorithm as a region favoring coiled-coil formation; indeed, the probability to form this structure is 1 with programs Coil and Paircoil. Likewise, zinedin and SG2NA have the same ability to form a coiled-coil structure. Such domains are implicated in protein dimerization and oligomerization. A well known example is the Gβ-Gγ complex of heterotrimeric G proteins (22). The coiled-coil partners of striatin, SG2NA, and zinedin are not yet known; their identification is pursued. They could perhaps help associate these proteins to membranes, in the way γ subunits of G heterotrimeric subunits direct the membrane attachment of β subunits (23).

Striatin has been the first member of the WD repeat family of proteins known to bind CaM in a Ca²⁺-dependent manner (1). Its CaM-binding site is a basic amphiphilic helix (4). Moreno et al. (8) showed that the 94-kDa species of SG2NA also binds CaM in a Ca²⁺-dependent manner. Indeed, the putative CaM-binding site of SG2NA differs from its counterpart in striatin by only one aa. Here, we show that the two 102- and 112-kDa species also bind CaM. A domain homologous to the CaM-binding sites of striatin and SG2NA is also present in zinedin but is significantly different in that it comprises two negatively charged aa instead of the uncharged aa glutamine and one proline residue instead of a glutamine (Fig. 7A). Endogenous brain zinedin, however, also binds CaM in a Ca²⁺-dependent manner. Whether the affinity of zinedin for CaM-Ca²⁺ is different from that of striatin is under investigation. Besides CaM, more and more neuronal Ca²⁺-binding proteins are being discovered (24). It may be that in vivo, the members of the striatin family are regulated by these proteins that, although similar to CaM, have different affinities for Ca²⁺.

Numerous signaling proteins, as well as proteins involved in cytoskeletal assembly and vesicular traffic, belong to the WD repeat superfamily (5). The WD repeat propeller structure, common to these proteins, creates a stable platform that reversibly interacts with several proteins either sequentially or simultaneously. We previously reported that the C-terminal moiety of the striatin sequence contains eight WD repeats (1). The first WD repeat, however, only weakly conforms to the consensus, and the variable region between the first two repeats, 6 aa long, is shorter than usual (5). The knowledge of the three dimensional structure of one of these WD domains would decide whether the proteins of the striatin family consist of seven or eight WD repeats. The WD domains of zinedin and SG2NA are homologous to that of striatin (Fig. 2A). They are,
however, the least conserved of the four domains, being, respectively, 61% and 70% identical to striatin. This is an indication that some of the binding partners of the WD repeats are different from one protein to the other.

A putative caveolin-binding domain corresponding to the consensus sequence described by Okamoto et al. (25) is present in the N-terminal region of all three members of the striatin family but not in Gβ subunits. A number of studies support the hypothesis that caveolin serves as a scaffold that brings together a variety of signal transducers. In striatin, the sequence encoding the caveolin-binding motif is FLQHEWARF (the three aromatic aa that are essential are underlined). In a preliminary study, we have shown, by directed mutagenesis, that the caveolin-binding domain of striatin is functional (3). The similar domains in zinedin and SG2NA should be functional too, because their sequences differ from that of striatin by one aa of the same class (tyrosine in SG2NA versus phenylalanine in striatin and zinedin, isoleucine in zinedin, and SG2NA versus leucine in striatin; Fig 2B). Because striatin, zinedin, and SG2NA are not transmembrane proteins, their binding to caveolin could be one of the probably many ways by which they associate with the cytoplasmic face of membranes. The members of the striatin family are mostly expressed in the brain, and the presence of caveolin in neurons has long been contended. That caveolin is expressed in neurons has now been established (26).

In summary, striatin, zinedin and, the two isoforms of SG2NA described here (SG2NA and SG2NAβ) bear homologous protein-protein interaction domains that are organized and spaced in the same way along the peptide sequence. They thus constitute a multigenic family of proteins.

This assumption is reinforced by the fact that similar proteins are present in metazoa throughout their evolution (18). The structural identity of the human SG2N and STRN genes adds credibility to the hypothesis that the two genes arose from an ancient duplication. This would be consistent with the current hypothesis about chordate genome evolution, which proposes that two rounds of large scale duplication occurred just before the emergence of bony fish (27). The human genes encoding the three members of each of the striatin, Na⁺-Ca²⁺ exchanger, and CaM families have been localized to the same loci, suggesting that these paralogous regions of the human genome have conserved synteny throughout the tetraploidization events (28). It is of interest to note that in each of the latter families, only three genes are found in the human genome, suggesting that a fourth gene, which should have arisen from the tetraploidization, has been deleted.

Analysis of the expression of the C. elegans paralogous gene showed that the protein is expressed in the worm muscles and in several neurons. In mammals, striatin and zinedin are mostly expressed in the brain, and SG2NA is expressed in brain and muscle. Our study revealed that there are at least two isoforms of mammalian SG2NA, 713 aa (SG2NA) and 797 aa (SG2NAβ), resulting from an alternate splicing. Other isoforms may exist, because we regularly detect three proteins by Western blotting. SG2NA was originally assumed to be a membrane-bound protein. Our study revealed that there are at least two isoforms of mammalian SG2NA (but not zinedin, isoleucine in zinedin, and SG2NA versus leucine in striatin; Fig 2B). Because striatin, zinedin, and SG2NA are not transmembrane proteins, their binding to caveolin could be one of the probably many ways by which they associate with the cytoplasmic face of membranes. The members of the striatin family are mostly expressed in the brain, and the presence of caveolin in neurons has long been contended. That caveolin is expressed in neurons has now been established (26).

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We have compared the distribution of rat striatin and SG2NA (but not zinedin) in sections of aldehyde-fixed rat brains. We found that the two proteins are often, if not generally, expressed in different types of neurons. In the striatum, striatin was strongly expressed in all spiny efferent neurons (29), whereas SG2NA immunoreactivity was present in the large, aspiny interneurons. In the cerebellum, Purkinje cells were strongly immunostained by anti-SG2NA antibodies but not by anti-striatin antibodies. It is interesting to note that in adrenal glands, the medulla, of neural origin, but not the cortex, was strongly immunolabeled by anti-SG2NA antibodies but not by anti-striatin antibodies. At the subcellular level, immunocytochemistry showed that both striatin and SG2NA were present in dendrites but not in axons. This identical, polarized distribution suggests that both proteins are addressed by the same mechanisms to their proper locations.

We have previously shown that cultured embryonic motoneurons in which striatin synthesis was specifically blocked have a reduced number of poorly branched dendrites, whereas the growth of axons appeared normal (6). We also showed that striatin plays a role in the control of locomotion. Indeed, the transient and specific down-regulation of striatin in the striata of behaving rats was paralleled by a sizable decrease in the locomotor activity of rats (6). If present in the cells deprived of striatin, SG2NA and/or zinedin did not functionally replace striatin. To approach the respective roles of the three proteins, the challenge is now to identify the specific partners of each of these proteins.

Very recently, Moreno et al. (8) put forward the challenging proposal that striatin and SG2NA might be a new class of B regulatory subunits of PP2A. These authors raised antibodies against a short peptide common to B’ subunits, and showed that such antibodies recognized both striatin and SG2NA, although the sequence homologies were very low. Using this antibody, they showed that, provided that the NIH-3T3 cells were reconstituted by the authors, a direct interaction between the PP2A C subunit and striatin or SG2NA has yet to be demonstrated.

Acknowledgments—We are indebted to Marc Bartoli, Yves Quentin, Pierre Pontarotti, and Jean-Pierre Kessler, who identified immunolabeled brain cells. We acknowledge RZPD, the Resource Center of the German Human Genome Project at the Max-Planck-Institut for Molecular Genetics as being the source of the EST clones used here.

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doi: 10.1074/jbc.M909782199 originally published online March 21, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M909782199

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