A Novel Calmodulin-binding Protein, Belonging to the WD-Repeat Family, Is Localized in Dendrites of a Subset of CNS Neurons

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Abstract. A rat brain synaptosomal protein of 110,000 Mr present in a fraction highly enriched in adenylyl cyclase activity was microsequenced (Castets, F., G. Baillat, S. Mirzoeva, K. Mabrouk, J. Garin, J. d'Alayer, and A. Monneron. 1994. Biochemistry. 33:5063–5069). Peptide sequences were used to clone a cDNA encoding a novel, 780-amino acid protein named striatin. Striatin is a member of the WD-repeat family (Neer, E.J., C.J. Schmidt, R. Nambudripad, and T.F. Smith. 1994. Nature (Lond.). 371:297–300), the first one known to bind calmodulin (CaM) in the presence of Ca+++. Subcellular fractionation shows that striatin is a membrane-associated, Lubrol-soluble protein. As analyzed by Northern blots, in situ hybridization, and immunocytochemistry, striatin is localized in the central nervous system, where it is confined to a subset of neurons, many of which are associated with the motor system. In particular, striatin is conspicuous in the dorsal part of the striatum, as well as in motoneurons. Furthermore, striatin is essentially found in dendrites, but not in axons, and is most abundant in dendritic spines. We propose that striatin interacts, through its WD-repeat domain and in a CaM/Ca+++-dependent manner, with one or several members of a surrounding cluster of molecules engaged in a Ca+++-signaling pathway specific to excitatory synapses.

Synapses are exquisitely organized subcellular structures (5). In the presynaptic compartment, the storage of neurotransmitters in vesicles, their release upon Ca++ influx, and their reuptake involve a host of proteins that include ion channels, enzymes, cytoskeletal elements, regulatory proteins, and transducers (44). In type I synapses, the postsynaptic compartment is characterized by the presence of a postsynaptic density (PSD), a dense, filamentous structure linked to the inner face of the postsynaptic membrane (for review see 20).

We have shown that mammalian brain synapses contain an adenylyl cyclase of unusually high specific activity (31). This has led us to speculate that either synapses contain an as yet unknown type of adenylyl cyclase, or that they are endowed with novel types of transducers or supramolecular complexes particularly efficient in the channeling of stimulatory signals to adenylyl cyclase. To test these possibilities, we have characterized some of the proteins present in synaptosomal preparations enriched in adenylyl cyclase. Among them, a protein of 110,000 Mr, yielded, by proteolytic digestion, peptides of unknown sequences. This study reports the cloning of the corresponding cDNA that encodes a novel protein, striatin, shown to directly bind calmodulin in a Ca+++-dependent manner. A member of the WD-repeat family of proteins (29), striatin is the first member of this family known to bind calmodulin. Subcellular fractionation studies indicate that striatin is a membrane-associated, Lubrol-soluble protein.

Striatin is shown here to be exclusively expressed in the central nervous system (CNS), principally in the neurons of the motor system: layer V of the sensory motor cortex, brain motor nuclei, especially the dorsal part of the caudate nucleus, and motoneurons, but also in the olfactory tubercle as well as, to a lesser extent, in the hippocampus. This striking distribution goes together with a mostly dendritic subcellular localization, especially dendritic spines, at the exclusion of axons. Striatin is therefore likely to engage in multiple protein interactions in a Ca+++-signaling pathway specific to postsynaptic compartments of a defined subset of CNS neurons.
Materials and Methods

Obtaining of Peptides and Microsequencing

Pooled fractions enriched in rat CNS synaptosomal adenyl cyclase (eluates from forskolin and calmodulin-affinity chromatographies, referred to as FC fractions in reference 6) were subjected to PAGE (7.5% SDS-polyacrylamide preparative gel). A piece of gel containing 110 (± 10) 45-kD proteins was cut out and digested with 2 μg/ml of endoproteinase Lys-C (Boehringer Mannheim GmbH, Mannheim, Germany) for 18 h at 30°C. The digested material was injected onto a DEAE column linked to a C 18 reversed-phase HPLC column. Eluted peptides were sequenced on a gas-phase sequencer (470; Applied Biosystems, Foster City, CA). All 10 peptides sequenced (KxELTDIAVLDNPK, KxVIDTSTRVK, KxWNPK, KMWNKOKxLaexAGAGALAK, KxEGAWETFT, KxTPGK, xPAKxAVVQGK, KxNFSYQEPK, and KxKSLRTGQDK) were not found in data banks.

Amplification of DNA Using Reverse Transcription–PCR

Total rat brain RNA was prepared by the urea-LiCl method (3). mRNAs were purified on oligo(dT)-cellulose spin columns (Clontech, Palo Alto, CA). Rat brain cDNAs were obtained by reverse transcription, using avian myeloblastosis virus reverse transcriptase and random hexanucleotides according to the manufacturer’s protocol (Amersham Intl., Little Chalfont, England). Using the cDNAs as templates, PCR was achieved with degenerate oligonucleotide primers derived from the sequences of five of the peptides obtained from the protein of 110,000 M., in the presence of Taq polymerase (Eurobio, Les Ulis, France). A 0.8-kbp DNA fragment was reproducibly obtained using the sense primer corresponding to the sequence YIVLDNKF: 5′-ACGGATCCAT(C,T)GT(A,C,G,T)CTCTT(A,C,G,T)GA(C,T)AATTCCACAT(T,C)TT-3′, and the antisense primer corresponding to peptide KMWNLQ: 5′-AGATGC- GACTG(A,C,T) A(A,G)TA(T,C)CCAT(T,C)TT-3′. The 0.8-kbp amplified DNA fragment was cloned in plasmid pUC18, yielding pUC18 FC used to generate a probe with which to screen a cDNA library.

Cloning of the cDNA Encoding Striatin

Among three rat brain cDNA libraries analyzed by PCR screening using a cDNA fragment obtained with oligonucleotides internal to the 0.8-kbp insert (5′-CAGGTGATAGACCCAGGATG-3′ and 5′-CCCTACTGATGTCTGCCTC-3′), one, constructed in X ZAP II (randomly primed; a gift of Dr. Marius Sudol, Rockefeller University, New York), was positive. Escherichia coli Y 1090 were infected with this library, and filter replicas (2 × 106 phage plaques) were hybridized using the 0.8-kbp insert from pFCC as template and the following oligonucleotides: 5′-ATGGATC-CTTCTAA -3′ and 5′-ATAAGCTTAAACA -3′. The 0.7-kbp KpnI-HpaI fragment of pFC5, which contains the 5′ part of striatin, the so-called complete fusion protein, pFCX, used to generate a fusion protein containing the first 50 amino acids of striatin, called pFCX, was constructed in X ZAP II (randomly primed; a gift of Dr. Marius Sudol, Rockefeller University, New York), was positive. The 0.8-kbp amplified DNA fragment was cloned in plasmid pUC18, yielding pUC18 FC used to generate a probe with which to screen a cDNA library.

Figure 1. Cloning of striatin cDNA and constructions of expression vectors. (Solid lines) Striatin cDNA sequence. (Boxes) Flanking plasmid sequences. pFCC 5, 6, and 9 are three of the fourteen isolated pBluescript clones containing striatin inserts. pFCC is a full-length clone constructed from pFCX and pFCC 5 and pF policeman and pF DNA result from the insertion into pSVL and pDNA 3 of the appropriate pFCC fragments. pFCX was obtained by digestion of pFCC.

The 0.3-kbp KpnI–HpaI fragment of pFCC, which contains the 5′ part of the coding sequence, was inserted into pC9 previously deleted from its KpnI–HpaI fragment, yielding pFC encoding striatin (Fig. 1).

Construction of Prokaryotic Expression Plasmids

The pMF plasmid (Fig. 1) was constructed by inserting the first 150 bp of striatin cDNA into the BamHI and HindIII linearized plasmid pLC24 in frame, with the coding sequence of the NH2-terminal portion (first 98 residues) of bacteriophage MS 2 polymerase (3). The 150-bp fragment was obtained by PCR using pFCC as template and the following oligonucleotides: 5′-ATGGATCTTCTAA -3′ and 5′-ATAAGCTTAAACA -3′. The 0.8-kbp amplified DNA fragment was cloned in plasmid pUC18 yielding pUC18 FC used to generate a probe with which to screen a cDNA library.

DNA Sequencing

Recombinant plasmids were purified by alkaline lysis (4) and sequenced. The inserts were sequenced on both strands by the dideoxy method of Sanger, according to Garoff and Ansorge (14), using T7 DNA polymerase (Pharmacia, Uppsala, Sweden) or Sequenase (United States Biochemical Corp., Cleveland, OH) and [γ-32P]dCTP. T3 and T7 primers (Pharmacia), as well as primers defined by the OLIGO program (37) for the walking procedure, were used. Sequences were analyzed with the UWGCG program on a Silicon Graphics station (Mountain View, CA). The BLAST algorithm (2) was used for homology search on the GenBank data base.

Construction of Plasmids

Construction of the Plasmid Encoding the Full-Length Striatin Sequence.

DNA and RNA Blot Hybridization

Southern blots of rat genomic DNA digested by EcoRI, HindIII, BamHI, and PstI were carried out as described (40), using a probe obtained by PCR, spanning sequence 1,293-2,316 (which contains an EcoRI site at bp 1955), and labeled by random priming with [γ-32P]dCTP (Amersham Intl.). Hybridized DNA and RNA blot filters were washed at 0.1% SSC, 0.1% SDS at 68°C. The filters were exposed to X-ray films and subjected to autoradiography.

The Journal of Cell Biology, Volume 134, 1996 1052
double-headed arrow), 720-1,215, 1,293-2,316, and 1,970-2,685. The fragments were purified using a GeneClean kit (Bio 101, LaJolla, CA). 25 ng of each was radiolabeled (2 × 10^6 cpm/µg DNA) by random priming using a Stratagene kit in the presence of [α-32P]dCTP. An actin probe (M. Buckingham, Institut Pasteur, Paris, France) was radiolabeled in the same manner. Formalin-fixed, paraformaldehyde-dehydrated, defatted, and apposed to films (X-OMAT AR; Eastman Kodak, Rochester, NY; 365 pb) were obtained as follows: pFC 10, encoding sequence 840-1,192, and pFCX (Fig. 1) were linearized and transcribed using T3 and T7 polymerases (RNA transcription kit from Stratagene) in the presence of 50 μCi [35S]UTP/μl, 10 μCi/ml (Amersham Int'l.) in a final volume of 20 μl.

**In Situ Hybridization**

Four adult Wistar rats (200-250 g) were used. The animals were killed by decapitation, and the brains were quickly removed, frozen on powdered dry ice, and stored at −70°C. Frontal and sagittal sections of 10 μm were cut on a cryostat and thaw-mounted on subbed slides. In situ hybridization histochemistry was performed as described (38). Briefly, sections were fixed in 4% paraformaldehyde, acetylated, dehydrated, and embedded in paraffin. Antisense and sense 35S-radiolabeled RNA probes were then washed at 52°C in 50% formamide and 2× SSC and incubated with RNase A (100 µg/ml) in 2× SSC at 37°C for 30 min. Sections were dehydrated, defatted, and apposed to films (X-OMAT AR; Eastman Kodak, Rochester, NY) for 6-10 d. The 35S-radiolabeled RNA probes were obtained as follows: pFCX (Fig. 1) were linearized and transcribed using T3 and T7 polymerases (RNA transcription kit from Stratagene) in the presence of 50 μCi [35S]UTP/μl, 10 μCi/ml (Amersham Int'l.) in a final volume of 20 μl.

**Tissue and Cell Fractionation, Calmodulin-Sepharose Affinity Purification, Western Blots, and Immunoprecipitation**

Protein was determined by the method of Schaffner and Weissmann (41). Homogenates of rat whole brain, striatum, hippocampus, hypothalamus, cerebellum, and thalamus were heated in Laemmli sample buffer, equal amounts of protein were electrophoresed on 7.5% SDS-PAGE gels and blotted as described (31). Other types of PAGE described by Neville
Expression of Striatin in COS-I Cells

The Journal of Cell Biology, Volume 134, 1996 1054

**Expression of Striatin in E. coli Cells**

pMF was used to direct the synthesis of fusion protein I in E. coli SG 4044 cells grown at 41°C. The fusion protein was produced as inclusion bodies that were purified. They were dissolved in LSB sample buffer containing 2 ~Ci/ml of [83H]adenine (24 Ci/mmol; Amersham Intl.), and then centrifuged at 100,000 g for 70 min at 4°C. The supernatants were saved, and the pellets were homogenized in the presence of 0.9% Lubrol-PX (Lubrol/protein ratio of 2.5) and centrifuged as described (10). The Lubrol-soluble fractions were incubated for 4 h at 4°C with calmodulin (CaM)-Sepharose (~1 g per 10 µg protein) in the presence of 1 mM Ca ++, as described (9). The flow-through was saved. After several washes, the CaM-binding proteins were eluted in a 1 mM EDTA- or EGTA-containing buffer and TCA precipitated (9).

**Expression of Striatin in COS-I Cells**

COS-1 cells were cultured in DMEM (GIBCO BRL, Gaithersburg, MD) containing 10% FCS, 4 mM glutamine, and antibiotics; they were transfected either by the DEAE-dextran procedure (40) or by the liposome method (adapted from 36), using pSVL or pFSVL (5 µg per one 10-cm petri dish). Trypsinized COS-I cells were centrifuged, and the pellets were resuspended in a 1 mM EDTA- or EGTA-containing buffer and TCA precipitated (9).

**Immunochemistry**

15 young male Sprague-Dawley rats (120-140 g) were deeply anesthetized and perfused transcardially with 300 ml of a cold 3% paraformaldehyde solution containing 0.08% glutaraldehyde. Coronal Vibratome sections of the striatum were collected in ice-cold PBS containing 50 mM NH4Cl and cryoprotected before being permeabilized by three cycles of freeze-thawing in liquid nitrogen. The preembedding free-floating technique was used. Briefly, the blocked sections were soaked overnight in ice-cold solutions containing 1-3 µg/ml of affinity-purified antibodies directed against striatin peptides II and III (Fig. 1 B). Biotinylated goat anti-rabbit second antibody (Vector Laboratories, Burlingame, CA) was used at a dilution of 1:100 and decorated with avidin-biotin-HRP complex (Vector Laboratories). The sections were postfixed with 2% glutaraldehyde, and the peroxidase complex was revealed with 0.05% 3,3' DAB tetrahydrochloride and 1% H2O2. Sections were fixed in 1% OsO4, contrasted in 1% uranyl acetate, dehydrated, and flat-embedded in Durcupan (Fluka AG, Buchs, Switzerland). Ultrathin sections were observed without further staining on an electron microscope (CM 10; Philips Electronic Instruments, Mahwah, NJ). A few sections were processed for light microscopy just after the DAB reaction. Three purified anti-peptide antibodies raised against peptides II and III were used: two gave comparable immunocytochemical reactions, and one gave no reaction and was used as a control. Other controls included (a) the omission of the primary antibodies, and (b) the use of primary antibodies adsorbed with their peptides added at a 100:1 molar ratio.

**Results**

**cDNA Cloning of Striatin and Analysis of the Predicted, Encoded Protein Sequence**

A 110,000-Mr, Lubrol-soluble protein present in an adenylyl cyclase–enriched synaptosomal fraction was obtained by PAGE and digested in the gel. 10 of the obtained peptides were sequenced and found to be original. Sense and antisense degenerate oligonucleotides deduced from five of them were used as primers in reverse transcription–PCR reactions with cDNAs obtained by reverse-transcribed rat brain mRNAs as template. A novel 0.8-kbp DNA fragment was obtained, encoding four of the se-
quired peptides. The screening of a randomly primed, λ ZAP rat brain cDNA library with this probe yielded 14 overlapping clones, defining a novel 3,635-bp sequence. This sequence contains an open reading frame (ORF) of 2,343 nucleotides (nt) flanked by a 656-nt-long 5' untranslated sequence (UTR) and a 636-nt-long 3' UTR (Fig. 2, A and B). The 3' untranslated sequence does not contain a polyadenylation signal. The protein encoded by the ORF has been called striatin since, as shown below, it is predominantly localized in the striatum. An ATG codon, preceded by several stop codons, lies within a canonical eukaryotic translation start sequence (22). The predicted protein sequence encodes 780 amino acids. It contains five of the ten peptides originating from the digestion of the 110,000- Mr component. Thus, in the preparative gel, another synaptosomal protein migrates with an apparent relative molecular mass identical to that of striatin. We have sequenced the latter protein, which appears to be a glycosidase (to be reported elsewhere).

A search for protein homologies in the NCBI data bank shows the presence, at the COOH-terminal end, of eight short sequences known as β-repeats or WD units at positions 419–454, 461–491, 514–544, 567–597, 613–643, 662–692, 704–734, and 746–780 (Fig. 2 B, numbered from 1 to 8); striatin is thus a member of the WD-repeat family (29). One WD-unit perfectly matches the consensus (repeat 2), and four contain three or fewer mismatches (repeats 4, 6, 7, 8); unit 1 (six mismatches), 3 (4 mismatches), and 5 (8 mismatches) only loosely conform to the consensus (Fig. 2 C). Besides, numerous potential phosphorylation sites are scattered over the sequence: 10 for calcium/phospholipid-dependent protein kinase C (serine or threonine 180, 194, 382, 405, 458, 486, 511, 592, 695, and 729) and 18 for casein-kinases. Four highly charged short sequences, one bearing a net positive charge of 9 (331–353), and another bearing a net negative charge of 7 (246–258), are found in the NH2-terminal region. The region spanning sequence 87–104 is predicted to be an amphipatic α helix having a positively charged side and a hydrophobic side. Some of these structural elements are shown in Fig. 2 B. Analysis of the striatin protein sequence does not reveal hydrophobic α helices suggestive of transmembrane segments. No known ATP- or GTP-binding consensus sequences are present. Striatin has a calculated isoelectric point of 5.16.

While our study was completed, the cDNA of a new member of the WD-repeat family, coding for a shorter, 713-amino acid human nuclear protein, SG2NA, of unknown function, was published (28). Its protein sequence is 80% similar to and 66% identical with sequences of a similar length from striatin, in both the NH2-terminal and the WD-repeat COOH-terminal domains, but not in the central domain. Although Muro et al. (28) report six WD repeats in the SG2NA sequence, we indeed noticed in this sequence two more WD repeats, at positions 353–387 and 679–713. Having now obtained the human striatin sequence (to be published elsewhere), we can state that striatin and SG2NA are different proteins, defining a new subfamily of WD-repeat proteins.

**Striatin Is a Detergent-soluble 110,000-Mr, Protein**

To identify the product of the *striatin* gene, antisera were obtained. Some were raised against a fusion protein expressed in *E. coli* by plasmid pMF (Fig. 1), containing the first 30 amino acids of striatin (fusion protein I; Fig. 2 B); others were directed against two charged striatin peptides II (sequence 191–205) and III (sequence 267–287), located in the NH2-terminal region of the protein (Fig. 2 B). Immunoblot studies carried out with each type of anti-striatin antibodies reveal the presence of only one 110,000-Mr protein in rat brain and spinal cord homogenates as well as in synaptosomal purified membrane fractions where this protein is enriched (Fig. 3, A and B, lanes 2 and 1, respectively). Striatin has a theoretical molecular mass of 86,225. The apparent relative molecular mass of striatin deduced by PAGE is thus higher than predicted by 23,800. When
Striatin is analyzed on other gel systems, Neville SDS gels (30) or gels containing either 6 M urea or an anionic detergent instead of SDS (32). It again has a 110,000 Mr, confirming its anomalous electrophoretic behavior. Striatin is particulate, only ~1% being found in the 100,000-g supernatant of rat brain homogenates or synaptosomes. Upon Lubrol solubilization of brain or synaptosomal pellets, striatin mostly partitions in the Lubrol-soluble fractions. When synaptosomes are resolved in a PSD fraction and a Lubrol-soluble fraction (7), striatin is essentially present in the Lubrol-soluble fraction. Even in striatal homogenates, the richest source of striatin, this protein occurs in very low abundance, not being revealed by Coomassie blue even in overloaded gels. This fact is in agreement with the scarcity of the 110,000-Mr synaptosomal protein from which we obtained peptides.

**Expression of Striatin in Heterologous Systems**

To confirm the identity of the brain 110,000-Mr protein, we compared its electrophoretic behavior with those of the proteins expressed by striatin-encoding vectors in bacteria and mammalian cells. *E. coli* cells transformed with the plasmid pFCGN (Fig. 1) express a soluble, 110,000-Mr fusion protein (expected Mr: 89,200), migrating at the same position in SDS-PAGE as brain striatin (Fig. 3 A, lane 1). This is in favor of pFCGN being a full-length clone. It also follows that euarkyotic posttranslational modifications are not likely to account for the anomalous behavior of striatin in SDS-PAGE.

Mammalian COS-1 cells were transfected with plasmid pFSVL encoding striatin (Fig. 1). A 110,000-Mr protein, migrating in SDS-PAGE at the same position as brain striatin, can thereby be detected by anti-peptide antibodies I and III in Western blots of lysates or immunoprecipitates of the lysed cells (Fig. 3 A, lane 3).

**Striatin Directly and Quantitatively Binds Ca++-CaM**

Brain striatin quantitatively binds CaM-Sepharose in the presence of Ca++ (Fig. 3 B, lanes 1 and 2), but not in its absence. It is eluted from the affinity-resin by EDTA- or EGTA-containing buffers, as shown by Western blots (Fig. 3 B, lane 3).

The bacterially expressed fusion protein comprising the full-length striatin also quantitatively binds CaM-Sepharose in the presence of Ca++ (Fig. 3 C, lanes 1 and 2), but not in its absence, and is eluted from the resin by EDTA- or EGTA-containing buffer (Fig. 3 C, lane 3). Since calmodulin is not found in bacteria, and since bacterial proteins do not bind CaM-Sepharose in a Ca++-dependent manner, this observation demonstrates that striatin directly binds Ca++-calmodulin.

**Striatin Is Not Directly Involved in cAMP Synthesis**

Since striatin was discovered in synaptosomal adenyl cyclase–enriched fractions, various studies have been performed to establish whether or not this protein is involved in cAMP production, by itself or by interacting with adenyl cyclase components.

The bacterial full-length fusion protein expressed by plasmid pFCGN is unable to restore a cya phenotype in *E. coli* cells deficient in adenyl cyclase, whereas these cells harbor a plasmid encoding calmodulin or not. The bacterially expressed striatin thus does not encode an adenyl cyclase able to function in *E. coli*.

Pellets of immunoprecipitated brain striatin do not contain any measurable adenyl cyclase activity. Brain FC fractions highly enriched in adenyl cyclase activity, when preincubated with affinity-purified anti–peptide I and II antibodies in large excess, display the same adenyl cyclase activities as do control fractions (not shown). Likewise, FC fractions complemented with various amounts of bacterially expressed striatin (contained in CaM-Sepharose EDTA eluates, as described) have the same adenyl cyclase activities as control fractions.

We have compared the cAMP contents of COS-1 cells overexpressing striatin (pFSVL-transfected cells) and of control cells, according to the procedure described by Salomon (39). Various assay conditions were tested: basal conditions (cells unchallenged with adenyl cyclase effectors), stimulatory conditions (addition of 10 μM forskolin, with or without 1 μM isoproterenol, to the culture medium), and assays in the presence of various concentrations of the Ca++ ionophore A 23187. No meaningful differences in the cAMP contents of control and transfected cells could be detected (not shown). We conclude that the overexpressed striatin does not grossly modify the cAMP synthesis machinery of COS cells. In those cells, however, the type(s) of adenyl cyclase expressed is not known, and it is conceivable that striatin, in case it affects the function of adenyl cyclase, operates in the context of one particular type of adenyl cyclase.

**Striatin Is Mostly Expressed in the Central Nervous System**

RNA analysis by Northern blots using four different probes, which together span the whole striatin coding sequence, show that among all tested rat or mouse tissues, striatin is expressed almost exclusively in the central nervous system (Fig. 4 A, lanes 1, 2, 3, and 11). Four rat transcripts of 8.3, 7.7, 5.5, and 4.7 kb are detected. Their lengths greatly exceed that of the striatin ORF (2.3 kb); however, brain messenger RNAs are known to have unusually long 3’ UTRs (43). The 8.3- and 5.5-kb transcripts are most abundant in brain and cerebellum (Fig. 4, lanes 1 and 2), the 4.7 transcript is the major species in the spinal cord (Fig. 4, lane 3). Blots exposed for longer times reveal all four transcripts in testes (not shown). In Northern blots of mouse brain revealed with rat probes, only two transcripts of 8.3 and 5.5 kb are readily detected (Fig. 4 A, lane 11). Only two of the four transcripts encode the full-length protein, as shown in Northern blots hybridized with a probe corresponding to the 5’ region (probe 1; Fig. 2 B, double-headed arrow).

To know whether the four transcripts originate from one or several homologous genes, rat genomic Southern blots have been probed with a DNA probe revealing the four transcripts. They yield a simple pattern, only one fragment being present in the DNA digested with BamHI or PstI (Fig. 4 C, lanes 1 and 4) and two with EcoRI (Fig. 4 C, lane 2), as expected if the four transcripts originate from one gene.
found in striatal homogenates (Fig. 3 D, lane 4). Striatin is present but less abundant in cortex, hippocampus, and cerebellum (~10 times less per mg protein;Fig. 3, lanes 2, 3, and 5), and scarce in the hypothalamus (Fig. 3, lane 1). Immunocytochemical labeling of brain sections using affinity-purified antibodies directed against peptides II and III reveals that striatin is found in the striatum, where the perikarya of most neurons are labeled, as well as many elements of the neuropil (Fig. 6, A and B). Other structures that build up the motor system, i.e., the pyramidal neurons from layer V in the sensory-motor cortex, brain nuclei such as the red nucleus (to be shown elsewhere) as well as the cerebellum, in the molecular layer, are also labeled (Fig. 6 D). In spinal cord sections, striatin is seen in motoneurons, in cell bodies and dendrites (Fig. 6 E). Similar results have been obtained with the two sets of antibodies directed against different epitopes of striatin. Sections treated with antibodies preincubated with an excess of the relevant peptide do not show reaction product (Fig. 6 C).

At the ultrastructural level in the striatum, the vast majority of neurons are stained. These neurons have a rounded nucleus and all the morphological characteristics of medium spiny, GABAergic neurons. The subcellular structures that contain the highest amount of DAB reaction product are dendritic spines (Fig. 7, A, C, and D); labeled dendritic shafts are sometimes seen (Fig. 7 B). In conditions that limit the dispersion of the reaction product away from its origin, the immunolabeling is mostly localized in the vicinity of the postsynaptic density and of the postsynaptic membrane (Fig. 7 D). Dendrites are, however, labeled all along their course, whereas axons are unlabeled. In neuronal cell bodies, a discrete labeling occurs in the cytoplasm around the nucleus, but not in the nucleus itself or in the lumen of the rough ER or of the Golgi apparatus (not shown). Sections treated with antibodies preincubated with an excess of the relevant peptide do not show any reaction product (Fig. 7 E).

**Discussion**

Striatin, a novel protein found in the CNS, has several characteristics, suggesting a possible role in postsynaptic signaling. First, it directly binds CaM in the presence of Ca++, as demonstrated by the study of bacterially expressed striatin. Secondly, its sequence contains eight WD-repeat proteins is characterized by the presence of a series of four to eight conserved repeating units of 23–41 amino acids, usually beginning with GH and ending with WD, separated by short, variable sequences (29). To our knowledge, striatin is the first known WD-repeat protein that binds Ca++. Up to now, all known proteins belonging to this family are eukaryotic regulatory proteins, none so far being an enzyme. Some interact with components of the cytoskeleton, like coronin (11), or of vesicles, like Sec1 3p (33), or participate in supramolecular complexes involved in transcription or translation processes. A number of them, among which are the β subunits of heterotrimeric G proteins, play a role in signal transduction.

Striatin is not a member of already known WD-repeat subfamilies, since its WD-repeats are not similar to theirs in equivalent positions. In particular, it is not a member of the...
Figure 5. Striatin is predominantly expressed in the striatum. Dark-field autoradiograms of rat brain sagittal (A and B) and coronal (C and D) sections. In situ hybridization with the antisense RNA probe (A and C) shows an intense and specific labeling in the striatum (st; A and C) and the olfactory tubercle (ot; A and C) and a moderate staining of the nucleus accumbens (a; C) and cortex (cx; A and C), as compared to the sense labeling (C and D). The positive hybridization signal in the hippocampus (h; A) detected with the antisense probe is significantly stronger than the weak staining seen with the sense probe (B); the signals given by both probes are comparable for the cerebellum (cer; A and B).
Figure 6. Striatin is present in the CNS motor system. Immunocytochemical localization at the light microscope. Immunolabeling obtained with the affinity-purified anti-peptide III antibody. (A–C) Coronal sections of the striatum. (A) Low concentration of antibody (1 μg/ml). The striatum (str) is more labeled than the cortex (cx). No labeling is observed in the corpus callosum (cc). (B) Higher concentration of antibody (3 μg/ml). The perikarya of most striatal neurons are labeled, but not the nuclei. The neuropil is strongly labeled. (C) Adjacent section treated with the antibody preadsorbed with peptide III. (D) Coronal section of the cerebellum. The molecular layer (ml) is densely stained, whereas no labeling is seen in the granular layer (gl). The Purkinje cell bodies (P) are lightly labeled. (E) Cross-section of the spinal cord, same treatment as in D. vh, ventral horn. The motoneurons are stained at the level of the cell bodies and dendrites, whereas their axons (arrows) are not. Bars, 100 μm (10 μm in B).

β subunits of heterotrimeric G proteins. The WD-repeat domains of rat striatin and human SG2NA (29) are, on the contrary, conserved. Although radically differing in their subcellular localizations, and therefore probably in their functions, striatin and SG2NA thus constitute a novel subfamily of WD-repeat proteins.

The peptides whose sequences allowed us to clone striatin originated from a Lubrol-soluble synaptosomal protein thought to be membrane bound (6). The study of brain subcellular fractions with antibodies directed against striatin peptides and a fusion protein confirmed that striatin is almost entirely particulate and that most of it can be solubilized by Lubrol. Striatin is therefore not a protein intrinsic to PSDs, but appears to be associated with membranes. Yet, we found no sequences able to account for hydrophobic α helices or consensus sequences for lipidic, posttranslational modifications, although palmitoylation is theoretically possible. Striatin possesses two polybasic domains, one bearing a net positive charge of 9. They may play a role in the binding of striatin to membranes, since it has been shown that stretches of basic amino acids mediate or at least help to promote protein binding to the membrane (21).

Striatin is largely expressed in structures belonging to the CNS motor system. In situ hybridization and immunocytochemistry show that striatum is the brain structure displaying the highest content of striatin. The caudate nucleus contains significantly more striatin than the nucleus accumbens. In Huntington-affected striata, the neurons that undergo apoptosis are also those present in the caudate nucleus, not those in the nucleus accumbens. Besides
Figure 7. In the striatum, striatin is localized in the postsynaptic compartment of synapses. Electron micrographs. Immunocytochemical localization obtained with an affinity-purified antibody directed against peptide III. (Similar results were obtained with antibody directed against peptide II). (A) Low magnification showing immunolabeled dendritic spines (arrows) and one unlabeled synapse (star). (B) Higher magnification showing a labeled structure that is probably a dendritic shaft. In this experiment, both the first antibody (3 μg/ml) and DAB concentrations were intentionally increased to obtain a strong labeling. (C) A labeled dendritic spine. (D) Lighter labeling obtained at low first antibody (1 μg/ml) and DAB concentrations, suggesting that striatin is localized close to the postsynaptic density (arrowhead) and to the postsynaptic membrane in dendritic spines. (E) Absence of immunoreaction inside two dendritic spines. In this control, the affinity-purified antibody directed against peptide III was preadsorbed overnight with peptides III and II in a 100-fold molar excess before immunolabeling. Bars, 0.2 μm.
striatum, the brain cortex, cerebellum, spinal cord grey matter, olfactory tubercle, and hippocampus also contain striatin messengers. Immunolabeling studies of brain and spinal cord sections using affinity-purified anti-striatin peptide antibodies confirm the presence of striatin in the motor system, in particular layer V of the sensory-motor cortex, striatum, brain motor nuclei, cerebellum, and spinal cord grey matter (to be described in detail elsewhere). These studies indicate that (a) only neurons contain striatin, no glial cells having been shown to contain any. (b) In the labeled neurons, two compartments contain striatin: the cell bodies, with the exception of nuclei, and more abundantly, the neuropil. In the cerebellum, the molecular layer is immunolabeled, but not the granular layer. In spinal cord sections, the dendrites of the motoneurons are stained, whereas the axons are unlabeled. Ultrastructural studies show that the vast majority of striatal neurons contain striatin and have the morphological characteristics of medium spiny neurons. Striatin is mostly confined to dendrites, predominantly the dendritic spines, where the reaction product is apposed to the inner face of the synaptic membrane and to the PSDs. Thus, in all neurons examined so far, striatin is present solely in the somato-dendritic compartment, predominantly in the dendritic spines.

The high concentration, in dendritic spines, of components involved in cAMP and Ca++ signaling is generally viewed as facilitating the reception, transmission, and integration of signals carried by both cAMP and Ca++ (8, 26). Indeed, the postsynaptic compartment contains channels such as voltage-gated ion channels and glutamate (N-methyl-D-aspartate [NMDA]) receptor channels, regulatory enzymes, and proteins involved in transduction, such as the abundantly represented calcium/CaM-dependent protein kinase type II, cAMP-dependent protein kinase, CaM, CaM-dependent cAMP phosphodiesterases (23), calcium/phospholipid-dependent protein kinase C (1), calcineurin (17), and adenylyl cyclase (27). Although adenylyl cyclase is expressed in neurons in the same postsynaptic compartment as striatin, so far we have been unable to find a relationship between striatin and the cAMP machinery. Of interest, however, is the fact that striatum is the only one brain structure specifically endowed with adenylyl cyclase type V (16), in association with G(olf) C(12, 19) and Gbeta7 subunits (46).

The direct binding of striatin to Ca++-CaM must be of great importance, since dendritic spines are individual calcium compartments (47). The extremely large variations in Ca++ concentrations that occur within these structures (34) have numerous biological consequences, such as the local regulation of the ras and MAP kinase pathways (13) or of microtubules, or the mechanisms of Ca++ buffering, so important with respect to Ca++ cytotoxicity (42). Striatin is thus likely to interact, in a Ca++-dependent manner and through its WD-repeat domain, with one or several members of the surrounding cluster of signaling or cytoskeletal molecules present in dendritic spines. The as yet unknown function of striatin is currently being investigated by the indirect, two-hybrid technique. The special distribution of striatin within the CNS turns it into a marker for a defined subset of neurons, and more particularly, of their dendrites. As such, it might be instrumental to study striatin in the course of neurodegenerative diseases.

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