Interaction of Calmodulin with Striatin, a WD-repeat Protein Present in Neuronal Dendritic Spines*

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Striatin is a quantitatively minor protein belonging to the WD-repeat family of proteins, is a Ca$^{2+}$/calmodulin-binding protein mostly expressed in the striatum and in the motor and olfactory systems (Castets, F., Bartoli, M., Barnier, J. V., Baillat, G., Salin, P., Moqrich, A., Bourgeois, J. P., Denizot, F., Rougon, G., Calothy, G., and Monneron, A. (1996) J. Cell. Biol. 134, 1051–1062). Gener-ally associated with membranes, striatin is mostly found in dendritic spines where it is likely to play a role in Ca$^{2+}$-signaling events. In this paper, we characterize its calmodulin-binding properties. By using deletion mapping and site-directed mutagenesis, we identified the sequence located between amino acids 149 and 166 as the main calmodulin-binding site. The predicted corresponding peptide is potentially able to form a basic amphiphilic helix, as is often the case for many known calmodulin-binding sites. Calmodulin binding to striatin is Ca$^{2+}$-dependent, with half-maximal binding occurring around 0.5 μM free Ca$^{2+}$. In the presence of Ca$^{2+}$, the equilibrium dissociation constant of calmodulin/striatin fusion protein complex is 40 ± 5 nM. We also show that brain striatin subcellular localization, as studied by tissue fractionation, is Ca$^{2+}$-dependent, this effect being probably mediated by calmodulin. Our results are in agreement with the hypothesis that striatin is a transducer involved in Ca$^{2+}$ signaling or an adapter protein involved in regulating macromolecular assemblies within dendritic spines.

Striatin is a quantitatively minor, Ca$^{2+}$/calmodulin (Ca$^{2+}$/CaM$^{1}$)-binding protein of $M_\text{r}$ 86,225, mostly expressed in neurons of the motor and olfactory structures of the central nervous system (1, 34). Cloning of the human homologous gene revealed that it is a highly conserved protein (96% identity, 96% similarity) (35). The C-terminal half of the protein sequence consists in a series of 8 WD repeats. WD repeats are found in a number of transducing proteins, the prototype of which is the β-subunit of heterotrimeric G proteins, and in proteins assisting in the assembly of multiprotein complexes (2). In synaptosomal preparations, striatin behaves as a membrane-associated protein, although its peptide sequence does not display hydrophobic stretches able to account for transmembrane domains nor signatures for myristoylation or farnesylation.

Striatin is exclusively expressed in the somatodendritic compartment of neurons, at the exclusion of axons. It is mostly found in dendritic spines, which are the postsynaptic compartments of excitatory synapses. Spines are tiny protrusions that stud the surface of dendrites (3). They are dynamic structures, their number and location varying with the ongoing neuronal activity (4, 5). Ca$^{2+}$ plays a major role in these structures, which are considered to be independent subcellular Ca$^{2+}$ compartments (5–7). They contain glutamate N-methyl-n-aspartic acid receptors, which are major Ca$^{2+}$-ionophores (8). Not surprisingly, they also contain large amounts of CaM (9) and CaM-binding proteins, principally Ca$^{2+}$/CaM kinase type II and, in significant amounts, calcineurin, adenylyl cyclases, and cAMP phosphodiesterases (reviewed in Refs. 3 and 10), as well as cytoskeletal proteins (myosin, fodrin 1, α-actinin) (11, 12).

Striatin, a component of spines, has molecular features suggesting that it, too, is involved in Ca$^{2+}$-signaling events. We have shown previously that striatin directly interacts with CaM in the presence of Ca$^{2+}$ (1). In this study, we mapped the CaM-binding site of striatin and determined its affinity for CaM. We also show that Ca$^{2+}$, most probably through its interaction with CaM, induces a partial subcellular redistribution of striatin. Our results are in agreement with the hypothesis that striatin is an adapter or transducer protein involved in Ca$^{2+}$/CaM-dependent events within dendritic spines.

Experimental Procedures

Construction of Expression Plasmids—Plasmids used to produce fragments of striatin fused to the C terminus of glutathione S-transferase (GST) were constructed using the pGEX-KT expression vector (13). First, plasmid pGEX-NcoI, with the new unique restriction sites NcoI, Ndel, and KpnI, was constructed by inserting an oligonucleotide (5′-GATCCATTGGGACATATGGGTACCG-3′) and its complementary strand (5′-ATTCCGTTACCATTATGGTACCG-3′) between the BamHI and EcoRI sites of pGEX-KT. The 1.95-kilobase pair NcoI-EcoRI fragment encompassing codons 1–650 of striatin (obtained from plasmid pFCC, see Ref. 1) was inserted between the corresponding sites of pGEX-NcoI, yielding pGEX-striatin 1–650. Plasmid pGEX-striatin 1–427, which codes GST fused to the first 427 residues of striatin, was constructed from pGEX-striatin 1–650: the plasmid was digested by StuI and EcoRI, blunted with T4 DNA polymerase in the presence of the four dNTPs, and ligated (the EcoRI site is restored upon ligation). Plasmid pGEX-striatin 1–98, which codes GST fused to the first 98 residues of striatin, was constructed similarly by deleting the BglII-EcoRI fragment of pGEX-striatin 1–650. To produce a fusion protein encoding the whole striatin sequence, the NcoI BamHI striatin fragment obtained from pFCC (in which a BamHI site immediately follows the STOP codon) was inserted into the NcoI- and BglII-digested plasmid pGEX-striatin 1–427. The resulting plasmid, called pGEX-striatin 1–780, codes GST fused for the entire striatin coding sequence (Fig. 1). Expression and Purification of Fusion Proteins—The plasmids were transformed into the Escherichia coli XL-1 Blue strain. 200-ml cultures were grown at 37 °C for 48 h on a shaker at 150 rpm. After the induction with 0.3 mM isopropyl-β-D-thiogalactopyranoside, the synthesis of fusion proteins was allowed to continue for a further 2 h. Cells were harvested by centrifugation, washed twice with PBS buffer, and resuspended in 100 ml of extraction buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10 mM β-mercaptoethanol, 1% Triton X-100). They were disrupted by sonication and the cell debris was removed by centrifugation. The supernatant was precipitated with 2 volumes of acetone and, after washing with 70% ethanol, was resuspended in small amounts of PBS buffer. The fusion proteins were then further purified by affinity chromatography on glutathione-Sepharose 4B columns.
of bacterial cells were grown until 0.3 A600 was reached. 0.1 ml iso- 
propyl-β-D-thiogalactopyranoside was then added, and incubation went on 
for 12 h at 18 °C. Cells collected by centrifugation were resuspended in 
4 ml of lysis buffer (20 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM phen-
ylmalenesulfonl chloride). Cells were frozen at −70 °C, thawed, and 
disrupted in a French press. The lysate was centrifuged at 12,000 g, 
at 4 °C for 15 min. The 4-ml supernatant was mixed with 2 ml of a 50% 
glutathione-Sepharose 4B slurry (Amersham Pharmacia Biotech) pre-
viously equilibrated with lysis buffer and incubated for 2 h at 4 °C. The 
suspension was centrifuged 5 min at 500 × g and the unbound proteins 
discarded. The resin was washed twice with 20 ml of lysis buffer. Fusion 
proteins bound to the matrix were eluted with 3 ml of lysis buffer 
containing 10 mM reduced glutathione.

Site-directed Mutagenesis—To modify the first putative CaM-binding 
site of striatin 1–427, plasmid pGEX-striatin 1–427 was digested by 
BglII and ligated with the oligonucleotide GATCGC, resulting in the 
disruption of the dipropeptide Pro-Asp between Asp-97 and Leu-98, in the 
middle of the putative CaM-binding site 1. 88 RGGQENKDKPDPLVR-
RKKML 107. The resulting protein is called striatin 1–427 mut 1. To 
modify the second putative CaM-binding site of striatin 1–427, a mod-
ified recombinant chain reaction was used for mutagenesis (14). Briefly, the primers 5′ CAGCAGAACACGGCATTCTAGG*GGA-
GCTAGGCG 3′ and 5′ ATGATCACTGTCCTCTGCTGCTGCAC-
CTCGC 3′ were designed for recombinant polymease chain reaction. 
The positions of these primers are underlined. A insert of 193 bp in the 
Trp-155 was changed to Gly and Gln-158 was changed to Pro (the 
corresponding codons are indicated by an asterisk). The resulting se-
quence reads: 149 QNSQFM *AGGCCGGC, allowing to check for the expected mutations. To intro-
duce this mutation in the whole fusion protein, we constructed plasmid 
pGEX-striatin 1–780 mut 2 by inserting the XhoI fragment of pGEX-
striatin 1–780 in the XhoI-linearized pGEX-striatin 1–427 mut 2. The 
protein expressed by this plasmid is called GST-striatin 1–780 mut 2. 
All constructs were verified by sequencing.

Calmodulin Overlay—CaM was biotinylated using the immuno-
probed2 B biotinylation kit from Sigma. Purified fusion proteins were 
separated on SDS-polyacrylamide (7.5%) gels and transferred onto 
nitrocellulose. Non-specific binding sites were blocked with 2% non-fat dry 
milk in TBS buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl) for 1 h. The 
nitrocellulose sheets were then incubated in TBS buffer containing 1% 
dry milk and 50 mM biotinylated CaM (bCaM), for 12 h at 4 °C (a 1% 
milk solution is about 3 mM Ca2+). After three 5-min washes in the 
same buffer, the blots were incubated for 1 h at room temperature with a 
1:500 dilution of ExtrAvidin-peroxidase. The resulting biotinylated 
sites were visualized using a Schleicher and Schuell slot blotter Mimiford. 
Striatin was revealed by Western blotting using an anti-striatin serum 
(see above) and ECL detection. Precise quantification of striatin was 
achieved by densitometric analysis of the immunoreactive bands, using a 
Bio-Rad machine. The results were expressed as a ratio of CaM-
binding site 1/2.

Deletion Mapping of the CaM-binding Site(s) of Striatin—In 
a previous study, we showed that recombinant striatin expressed in E. coli 
yielded CaM in a Ca2+–dependent manner (1). To localize the CaM-binding site(s), we produced in E. coli various fragments of striatin and determined their CaM-
binding capabilities. To facilitate their purification, the various 
fragments were genetically fused to glutathione S-transferase. 
A schematic representation of the different constructs is shown in 
Fig. 1. The fusion proteins were produced in E. coli at a low growth 
temperature. In these conditions, the recombinant proteins 
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RESULTS

Deletion Mapping of the CaM-binding Site(s) of Striatin—In 
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A schematic representation of the different constructs is shown in 
Fig. 1. The fusion proteins were produced in E. coli at a low growth 
temperature. In these conditions, the recombinant proteins 
were found in the soluble bacterial extracts and could be
purified by a single chromatographic step on glutathione beads. Fig. 2A shows a SDS-polyacrylamide gel electrophoresis analysis of the purified preparations. The CaM binding properties of the various proteins were tested by a CaM-overlay procedure, in which the proteins were separated on SDS-gels, transferred to nitrocellulose, and probed with bCaM. Fig. 2B indicates that bCaM bound in a Ca"+-dependent manner GST-striatin 1–427 (lane 1) and GST-striatin 1–780 (lane 5) but not GST-striatin 1–98 (lane 4). In a different set of experiments, the various GST-striatin fusion proteins were tested for their ability to bind CaM-Sepharose in the presence of Ca"+.

Fig. 2 CaM overlay functional analysis of striatin fusion proteins. Various striatin fusion proteins (30–70 pmol each) were purified on glutathione beads and separated on SDS-polyacrylamide gels. A, silver staining of the gel. Lane 1, GST-striatin 1–427; lane 2, GST-striatin 1–427 mut 2; lane 3, GST-striatin 1–98; lane 5, GST-striatin 1–780; lane 6, GST-striatin 1–780 mut 2. Molecular weight markers are shown. B, an identical gel was blotted onto nitrocellulose and analyzed by CaM overlay in the presence of Ca"+. Lane 1, GST-striatin 1–427; lane 2, GST-striatin 1–427 mut 1; lane 3, GST-striatin 1–427 mut 2; lane 4, GST-striatin 1–98; lane 5, GST-striatin 1–780; lane 6, GST-striatin 1–780 mut 2. In the absence of Ca"+, there is no staining.
under “Experimental Procedures” (Fig. 6A). Whether the homogenization was performed in the presence of EGTA or of Ca\textsuperscript{2+}, the cytosol contained 25 ± 5% of the total protein, the Lubrol-soluble proteins 58 ± 5%, and the Lubrol-insoluble pellets 17 ± 3%. The relative amount of striatin in each fraction was assessed by scanning of Western blots (Fig. 6B). When the homogenate was prepared in the presence of 1 mM EGTA, striatin was present in highest amount in the Lubrol-soluble fraction (Fig. 6C, lane 2), but was also found in the cytosol fraction (lane 3) and in the Lubrol-insoluble fraction (lane 1). When the homogenate was made in the presence of 100 μM Ca\textsuperscript{2+} (lanes 4–6), the Lubrol-insoluble fraction did not contain any striatin (lane 6). Densitometric analysis of the autoradiograms showed that in the latter case, the striatin content of the cytosol was almost twice that measured in the absence of Ca\textsuperscript{2+} (Fig. 6C). The same experiments were repeated four times with the same results.

**DISCUSSION**

Striatin has been the first member of the WD-repeat family of proteins known to interact with the ubiquitous calcium sensor protein, CaM (1). The WD repeats have been identified in numerous signaling proteins, and it has been suggested that this motif is involved in their multiple interactions with target proteins, within a regulatory network. The function of striatin is currently unknown, but its ability to interact with CaM as well as the presence of WD repeats in its sequence suggest that this protein is involved in Ca\textsuperscript{2+}-dependent signaling at the postsynaptic membrane level. Recently, the β-subunit of transducin, a heterotrimeric G protein, and the prototype of the WD-repeat family, was also shown by Liu and colleagues to directly bind Ca\textsuperscript{2+}/CaM (21). The authors suggest that Ca\textsuperscript{2+}/CaM-binding to Gβ differentially modulates the interaction of Gβ with its multiple effectors. We hypothesize that CaM binding to striatin could similarly affect its interaction with its targets.

The CaM-binding domain of striatin, called domain or site 2, has been defined in this study by directed mutagenesis and expression of fusion proteins. It includes residues 149–166 and conforms to the frequently encountered CaM-binding motif consisting in a basic amphiphilic helix. In the CaM-binding peptide of myosin light chain kinase, which typifies this motif, two aromatic residues, separated by 12 amino acids, are responsible for anchoring the peptide to the N- and C-terminal halves of CaM, respectively (22). In the case of striatin, it is likely that the aromatic residues Phe-153 and Tyr-165, separated by 11 amino acids, anchor striatin to the N- and C-terminal halves of CaM. Some proteins have two or multiple CaM-binding domains, for example the Trp1 ion channels (18), the α-syntrophin (23), a CaM-stimulated phosphodiesterase (24), the NR1 subunit of N-methyl-D-aspartic acid receptors (25). The possibility that striatin possesses two CaM-binding domains appears unlikely since the GST-whole length striatin fusion protein no longer binds CaM when domain 2 is mutated. This mutation was designed in such a way as to decrease the ability of this region to form an α-helix and to prevent the hydrophobic interactions involved in CaM binding. In the case of the β-subunit of transducin, Liu and colleagues (21) determined that the CaM binding corresponded to residues 40–63. Interestingly, residues 53–63 belong to the first WD repeat of the seven such repeats that constitute most of the protein. The CaM-binding domain of the β-subunit of transducin is there-
fore part of the propeller-like structure revealed by crystallography (26, 27). In striatin, on the contrary, the CaM-binding domain is away from the WD-repeat domain.

Striatin, a quantitatively minor, membrane-associated protein, is predominantly found in the dendritic spines of a few subsets of neurons (1). The Ca$^{2+}$/CaM-concentration requirement for CaM binding of the many postsynaptic CaM-binding proteins is variable, reflecting the fact that the intracellular concentration of Ca$^{2+}$ within dendritic spines varies in a highly dynamic way (6). Here we show that the affinity of the brain striatin-CaM complex for Ca$^{2+}$ is 0.1–0.5 μM. Hence striatin reversibly binds Ca$^{2+}$/CaM, depending upon the fluctuations of Ca$^{2+}$ concentration in spines. We measured the $K_D$ value of the complex made up of a GST-striatin fusion protein and of Ca$^{2+}$/CaM. This $K_D$ is of the order of 40 nm, it falls in the same range as that measured for synthrophins (20–100 nm) (23) and one of the two CaM-binding sites of the N-methyl-D-aspartic acid glutamate receptors (R1 subunit) (73 nm) (25).

It has been shown in some types of neurons that, following elevation of intracellular Ca$^{2+}$ and the ensuing binding of CaM to its targets, a certain percentage of CaM and of CaM-binding proteins is redistributed from membrane to cytosol and vice versa (28–31). The translocating proteins would thereby cycle between various states of conformation and/or activity. Although the reality of these phenomena is beyond doubt, estimates of the percentage of translocated proteins under such circumstances are not quite reliable (29, 32). In this study, we compared brain striatin subcellular localization in tissue extracts realized either in the presence or absence of Ca$^{2+}$. In the absence of Ca$^{2+}$, striatin was present in all subfractions, including the Lubrol-insoluble fraction, containing postsynaptic densities (10) and cytoskeletal elements. In the presence of 100 μM Ca$^{2+}$, the contrary, striatin was no longer present in this fraction. Such an in vitro differential distribution of striatin is likely to be mediated by CaM, since striatin is not known to directly bind Ca$^{2+}$. It is tempting to imagine that in vivo a shift in striatin distribution would also occur as a result of Ca$^{2+}$ fluctuations. Such a study could perhaps be attempted, using immunocytochemistry at the electron microscope level, on NT2 fully differentiated neurons, theoretically able to establish synapses (Stratagene). However, at the stage at which synapses are said to occur, we found that the cells were aggregated, the neurites completely entangled, and no conclusion could be drawn.

The expression of striatin in only a few well defined subsets of neurons renders the elucidation of the physiological role of striatin a difficult task. Techniques aiming at decreasing its expression in neuronal cells are currently being used. Future research can now take advantage of the possibility to study neurons expressing high levels of striatin whose CaM-binding site has been disrupted. Indeed, the regulation by Ca$^{2+}$/CaM of the function of striatin will have to be appreciated, since this protein is expressed in structures in which neurodegenerative processes might well be related to Ca$^{2+}$-dependent excitotoxicity (33).

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