Striatin, a calmodulin-dependent scaffolding protein, directly binds caveolin-1

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Abstract Caveolins are scaffolding proteins able to collect on caveolae a large number of signalling proteins bearing a caveolin-binding motif. The proteins of the striatin family, striatin, SG2NA, and zinedin, are composed of several conserved, collinearly aligned, protein–protein association domains, among which a putative caveolin-binding domain [Castets et al. (2000) J. Biol. Chem. 275, 19970–19977]. They are associated in part with membranes. These proteins are mainly expressed within neurons and thought to act both as scaffolds and as Ca^{2+}-dependent signalling proteins [Bartoli et al. (1999) J. Neurobiol. 40, 234–243]. Here, we show that (1) rat brain striatin, SG2NA and zinedin co-immunoprecipitate with caveolin-1; (2) all are pulled down by glutathione-*S*-transferase (GST)–caveolin-1; (3) a fragment of recombinant striatin containing the putative caveolin-binding domain binds GST–caveolin-1. Hence, it is likely that the proteins of the striatin family are addressed to membrane microdomains by their binding to caveolin, in accordance with their putative role in membrane trafficking [Baillat et al. (2001) Mol. Biol. Cell 12, 663–673]. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Caveolin; Striatin family; CHAPS solubilization

1. Introduction

Striatin is an intracellular protein of 780 amino acids (aa) mainly found in the somato-dendritic compartment, including spines, of a few subsets of neurons [4,5]. It is distributed both in the cytosol and in the membrane fraction in roughly equal amounts. Striatin contains several protein–protein association domains: from the N- to the C-terminus, a putative caveolin-binding motif, a coiled-coil structure, a Ca^{2+}–calmodulin-binding site and a large WD-repeat domain [4,6]. Hence, striatin is thought to be a scaffolding protein functioning in a Ca^{2+}-dependent manner. Striatin is a neuronal protein, whose down-regulation, both in developing motoneurons and in live rats, has severe effects [2]. Striatin directly interacts with phocein, a protein involved in vesicular traffic [3]. Two other proteins, SG2NA (including its splice variants), which is an ubiquitous protein, and zinedin, mainly restricted to the nervous system, are respectively 80 and 75% similar to striatin, they share identical protein–protein interaction domains identically aligned [1]. In particular, the caveolin-binding motifs of SG2NA and zinedin are 91% identical and 100% similar to that of striatin. Striatin, SG2NA and zinedin constitute the striatin family.

Caveolins, integral membrane proteins of 21–25 kDa, are the main components of caveolae, specialized domains of rafts [7]. Caveolins have a hairpin topology, with two intracytoplasmic domains on both sides of a 33 aa long intramembrane loop. They are scaffolding proteins able to interact with specific lipids and with a great number of signalling proteins: Ras, serine–threonine kinases, several tyrosine kinases, G protein-coupled receptors, several G-protein α subunits, some downstream effectors of G proteins [8]. Caveolins have a scaffolding domain of 20 aa long, located in the juxtamembranous region of the N-terminal moiety. This domain binds caveolin-binding motifs of the type ΦXΦXXXXΦ, ΦXXXXΦXXΦ or ΦXΦXXXXΦXXΦ, where Φ is an aromatic residue and X any aa [9]. In some instances, one of the Φ is replaced by Leu [10]. Such motifs are present in almost all signalling proteins that bind caveolins [11–13].

Caveolae are centers of signal transduction [8]. Typical caveolae have not yet been identified in neurons, however, microdomains containing caveolins-1 and -2 are part of the immensely complex neuronal plasma membrane [14,15]. The presence of a consensus caveolin-binding domain in the N-terminal region of striatin (^{53}LHFLQHEWARF^{63}), which is well conserved in SG2NA and zinedin [1], suggests that the interaction between these proteins and caveolin is direct. To clarify this point, we realized co-immunoprecipitation and pull-down experiments, which indeed show that this interaction is direct and functional in vitro. Hence, caveolin-1 may directly regulate the function of the proteins of the striatin family, by concentrating them within caveolin-enriched domains.

2. Materials and methods

Unless otherwise indicated, all experiments took place at 4°C.

2.1. Subcellular fractionation of rat brain

Adult Wistar rats were anaesthetized and their brain homogenized (1 g wet weight in 10 ml) in Tris–saline buffer (TBS; 50 mM Tris–HCl, pH 7.4, 150 mM NaCl) containing 1 mM EGTA or 0.1 mM

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Abbreviations: TBS, Tris–saline buffer; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfate; GST, glutathione-*S*-transferase; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PS, Pansorbin

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$CaCl_2$ and 5 mM $MgCl_2$. The homogenate was centrifuged 1 h at 100 000×*g*, yielding a cytosol fraction (1.5 mg protein/ml) and a 100 000×*g* pellet. The latter was homogenized in 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfate (CHAPS)-buffer (50 mM Tris–HCl, pH 7.4, 500 mM NaCl, 7.5 mM CHAPS (Sigma, St. Louis, MO, USA), containing either 1 mM EGTA or 0.1 mM $CaCl_2$ and 5 mM $MgCl_2$) and centrifuged 1 h at 100 000×*g*. Supernatants were called CHAPS fractions (2 mg protein/ml).

2.2. Plasmid constructs

The pGEX-caveolin-1 full length plasmid was a gift of Dr. Andrew Quest (University of Chile, Santiago, Chile) [16]. The pGEX-P-striatin 1–427 plasmid was constructed by inserting two complementary oligonucleotides (5′-GATCTCTGGAAGTGCTCTTCCAGGGTCCG-C-3′ and 5′-GATCGCGGACCCTGGAAGAGCACTTCCAGA-3′) encoding the PreScission Protease site (Amersham Pharmacia Biotech, Piscataway, NJ, USA) into the *Bam*H-1 site of the pGEX-striatin 1–427 plasmid [5]. The sequence of the resulting plasmid was verified (ESGS, Evry, France). The pGEX-P-SG2NA-β 1–465 plasmid encoded glutathione-*S*-transferase (GST) fused to the mouse 465 first aa of the SG2NA-β isoform (accession number: AF307777) separated by the PreScission Protease site [1].

2.3. Purification of recombinant proteins

Expression of the GST fusion proteins in *Escherichia coli* XL1Blue strain (Stratagene, La Jolla, CA, USA) was induced by overnight incubation with 0.1 mM isopropyl β-D-thiogalactoside at 18°C. Cells were lysed using a French press. Fusion proteins contained in the soluble fraction were purified on glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech). Beads coated with GST or GST–caveolin-1 were extensively washed with TBS and used as such. Beads coated with GST–P-striatin 1–427 or GST–P-SG2NA-β 1–465 were incubated overnight at 4°C in TBS containing 1 mM DTT, 1 mM EDTA and 10 U of PreScission Protease per 50 ml of culture. The supernatants contained the striatin 1–427 fragment and the SG2NA-β 1–465 fragment, respectively.

2.4. Antibodies

Rabbit anti-caveolin-1 antibodies were from Santa Cruz Biotech (sc-894, Santa Cruz, CA, USA). Rabbit anti-striatin antibodies were affinity-purified anti-peptide antibodies directed against rat striatin aa 267–287 [4]. To obtain rabbit anti-SG2NA antibodies, 200 μg of purified SG2NA-β 1–465 was resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Protran membranes (Schleicher and Schuell, Keene, NH, USA). The strip containing the purified protein was blocked at room temperature for 45 min in TBS containing 5% non-fat dry milk, incubated for 5 h at room temperature in 40 ml of a 10-fold dilution of anti-SG2NA serum [1]. The washed strips were incubated for 10 min with 1 ml of 0.1 M glycine–HCl buffer, pH 2.5, then with 1 ml of glycine–HCl buffer, pH 2.2. Antibody-containing eluates were neutralized, complemented with 0.1% bovine serum albumin (BSA), concentrated in a Prodicon apparatus (Bio-Molecular Dynamics, Beaverton, OR, USA), and aliquots were frozen at −20°C. Rabbit anti-zinedin antibodies were described [1].

Antibodies were revealed using the enhanced chemoluminescence (ECL) procedure (Pierce, Rockford, IL, USA) or the alkaline phosphatase procedure, depending on the background produced in each case.

2.5. Co-immunoprecipitation

Co-immunoprecipitation assays were performed as described previously [3] with slight modifications. Briefly, 4 ml CHAPS fractions (diluted to 250 mM NaCl, 7.5 mM CHAPS) were incubated with, respectively, 140 μg of rabbit pre-immune immunoglobulins (Sigma), 140 μg of anti-caveolin-1, 140 μg of anti-striatin, 140 μg of anti-SG2NA and 140 μg of anti-zinedin antibodies, overnight at 30°C, with gentle agitation. Batches of 70 μl of a 10% suspension of Pansorbin (PS) cells (Calbiochem, San Diego, CA, USA), pre-incubated in TBS containing 1% BSA, were added to each 4 ml sample and further incubated for 4 h at 30°C. PS cells were centrifuged at 12 000×*g* and washed five times in TBS containing 7.5 mM CHAPS (TBS–CHAPS buffer; two times with 15 ml and three times with 1.5 ml), for 10 min at 30°C with gentle agitation. Cell pellets were boiled 5 min in sample buffer. Solubilized proteins were electrophoresed on 8 and 15% SDS-PAGE and transferred onto Protran membranes.

2.6. Pull-down experiments

Pull-down experiments were performed as described previously [3] with modification. Briefly, 200 pmol of GST–caveolin-1 or of GST were bound to 30 μl of glutathione–Sepharose 4B beads. Washed beads were incubated, with gentle agitation, overnight at 30°C with 300 μl of CHAPS fractions or 300 μl of cytosolic fractions, or for 5 h at 30°C with 150 μl of a 0.2 μM striatin 1–427 solution prepared in TBS–CHAPS buffer. After four washes in 1.5 ml TBS–CHAPS buffer for 10 min at 30°C, beads were treated as described above. To quantify the amounts of striatin pulled down in the presence (0.1 mM) or absence of Ca^{2+} (1 mM EGTA), densitometric analysis of the immunoreactive bands was achieved using a specially designed software (NIH Image 1.59).

3. Results

3.1. Anti-caveolin-1 antibodies co-immunoprecipitate rat brain striatin, SG2NA and zinedin

Brain caveolin-1 was efficiently solubilized by the zwitterionic detergent CHAPS, provided that a rather high ionic strength buffer, containing 500 mM NaCl, was used. Upon fractionation of brain homogenate, striatin, SG2NA and zinedin partitioned in both cytosol and CHAPS fractions (Fig. 1A–C, lane 1), whereas caveolin-1 was present only in the CHAPS fraction (Fig. 1D, lane 1). We looked for a possible association between caveolin-1 and rat brain striatin, SG2NA and zinedin by co-immunoprecipitation experiments using

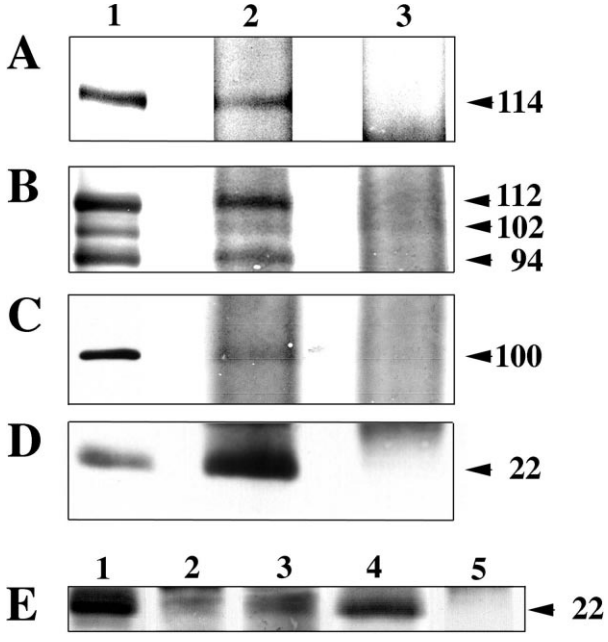

Fig. 1. Striatin, SG2NA and zinedin are co-immunoprecipitated with caveolin-1. A–D: PS cells, coated with anti-caveolin-1 antibodies (lane 2) or pre-immune immunoglobulins (lane 3), were incubated with CHAPS fractions (lane 1). The washed pellets were analyzed by Western blots using anti-striatin (A), anti-SG2NA (B) or anti-zinedin (C) affinity-purified antibodies, or anti-caveolin-1 antibodies (D). The ECL procedure for caveolin, and the alkaline phosphatase procedure, for striatin, SG2NA and zinedin were used. The apparent molecular weight (kDa) of the revealed proteins are indicated on the right (arrows). E: Caveolin-1 is co-immunoprecipitated with the three members of the striatin family. PS cells coated with anti-striatin (lane 2), anti-SG2NA (lane 3), anti-zinedin (lane 4) affinity-purified antibodies or pre-immune immunoglobulins (lane 5) were incubated with CHAPS fractions (lane 1). The washed pellets were analyzed by Western blots using anti-caveolin-1 antibodies and the alkaline phosphatase procedure. The apparent molecular weight of caveolin-1 is indicated on the right (arrow).

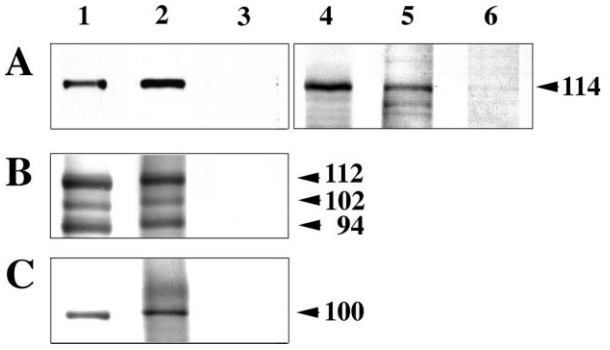

Fig. 2. GST–caveolin-1 pulls down endogenous rat brain striatin, SG2NA and zinedin. GST–caveolin-1-coated beads (lanes 2 and 5) and GST-coated beads (lanes 3 and 6) were incubated at 30°C with a CHAPS fraction (lanes 1–3) or a cytosol fraction (lanes 4–6). Washed beads were analyzed by Western blots using anti-striatin (A), anti-SG2NA (B) or anti-zinedin (C) affinity-purified antibodies. The ECL procedure, for striatin, and the alkaline phosphatase procedure, for SG2NA and zinedin were used. Apparent molecular weight of striatin, SG2NA isoforms and zinedin are indicated on the right (arrows).

CHAPS fractions and anti-caveolin-1 antibodies. Analysis of immunoprecipitates showed that striatin (Fig. 1A, lane 2), the isoforms of SG2NA (Fig. 1B, lane 2) and zinedin (Fig. 1C, lane 2) were co-immunoprecipitated along with caveolin-1 (Fig. 1D, lane 2) but were not present in immunoprecipitates obtained with control antibodies (Fig. 1, lane 3). Anti-striatin (Fig. 1E, lane 2), anti-SG2NA (Fig. 1E, lane 3) and anti-zinedin antibodies (Fig. 1E, lane 4) all co-immunoprecipitated caveolin-1 present in rat brain CHAPS fractions (Fig. 1E, lane 1).

3.2. GST–caveolin-1 pulls down rat brain striatin, SG2NA and zinedin

To further document the fact that rat brain striatin, SG2NA and zinedin interact with caveolin-1, we performed pull-down experiments using GST–caveolin-1 and CHAPS fractions. Rat brain striatin present in a CHAPS fraction (Fig. 2A, lane 1) or in a cytosol fraction (Fig. 2A, lane 4) specifically bound GST–caveolin-1 (Fig. 2A, lanes 2 and 5) but not GST (Fig. 2A, lanes 3 and 6). More striatin was pulled down when incubation took place at 30°C rather than 4°C. Interestingly, about 50 ± 10% less striatin (mean of three independent determinations) was pulled down by GST–caveolin-1 from Ca^{2+}- (Fig. 3, lane 4) rather than

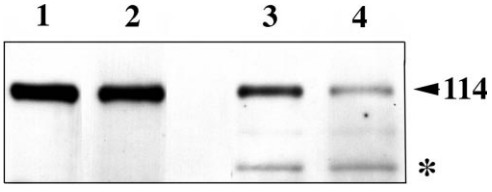

Fig. 3. The presence of free Ca^{2+} (0.1 mM) in CHAPS fractions (lane 2) decreases the interaction of striatin with GST–caveolin-1, as compared to EGTA-containing CHAPS fractions (lane 1). GST–caveolin-1 pulls down 50% less striatin from Ca^{2+}-containing CHAPS fractions (lane 4) than from EGTA-containing fractions (lane 3). The apparent molecular weight of striatin is indicated. The asterisk shows non-specific staining of a protein irrelevant to striatin.

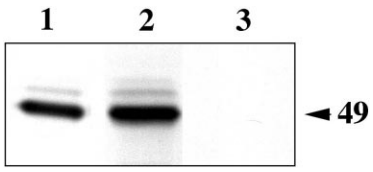

Fig. 4. Striatin directly interacts with caveolin-1. Washed GST–caveolin-1-coated beads (lane 2) and GST-coated beads (lane 3) were incubated with recombinant striatin 1–427 (lane 1) and analyzed by Western blots using anti-striatin affinity-purified antibodies. The ECL procedure was used. The apparent molecular weight of striatin 1–427 is indicated on the right (arrow).

from EGTA-containing CHAPS fractions (Fig. 3, lane 3). Rat brain SG2NA (Fig. 2B, lane 2) and zinedin (Fig. 2C, lane 2) also bound GST–caveolin-1 but did not bind GST (Fig. 2B,C, lane 3).

3.3. Recombinant caveolin-1 and striatin physically interact

The latter experiments do not indicate whether the interactions between caveolin-1 and rat brain striatin, SG2NA and zinedin are direct. To check this point, we purified a fragment of recombinant striatin containing the putative caveolin-binding domain, striatin 1–427 (Fig. 4, lane 1). Incubation of this fragment with GST–caveolin-1 and with GST showed that it specifically bound GST–caveolin-1 (Fig. 4, lane 2) but not GST (Fig. 4, lane 3).

4. Discussion

Caveolins have been reported to interact with an amazingly large number of signalling proteins [8]. Proteins for which a direct interaction with the scaffolding domain of caveolins has been demonstrated are fewer: endothelial and neuronal nitric oxide synthases [17,18], Ras [13], G protein-coupled receptor kinases 1, 2 and 5 [10], $p75^{NTR}$ [19], the androgen receptor [20]. The present study shows that striatin, SG2NA and zinedin all interact in vitro with caveolin-1, and that in the case of striatin, at least, they physically interact.

It has been only recently demonstrated that caveolin-1 and -2 are expressed in neurons [14]. Here, we have shown that brain caveolin-1 can be efficiently solubilized by CHAPS. To examine possible interactions between caveolin-1 and the proteins of the striatin family, we did not need to overexpress striatin, SG2NA or zinedin, since their amounts in brain fractions proved to be sufficient. To our knowledge, proteins of the striatin family are not modified by lipids. Many of the signalling proteins that interact with caveolin are known to be post-translationally modified by lipids [21–23]. However, in vitro studies have shown that such modifications are not a prerequisite for binding to caveolins [13].

Since pull-down experiments using GST–caveolin-1 and a fragment of striatin containing the caveolin-binding motif showed that the interaction of the two proteins is direct, and since the caveolin-binding motifs of SG2NA and zinedin are 100% similar to that of striatin, it is likely that the interaction of caveolin with SG2NA or zinedin is also direct. Because the caveolin-binding domain of the proteins of the striatin family is contiguous with the putative coiled-coil structure, a possibility would be that binding of caveolin-1 influences the state of this structure.

We have previously shown that the distribution of striatin

varies upon fractionation of brain homogenates according to their Ca^{2+} concentration: about twice as much striatin was found in the cytosol fraction from Ca^{2+}-containing homogenates than from EGTA-containing homogenates [6]. That the proteins of the striatin family also shift from membranes to cytosol according to local Ca^{2+} fluctuations in vivo is a likely hypothesis. In that case, at least some of their partners should change. Our finding that recombinant caveolin-1 pulls down less striatin in the presence of Ca^{2+} than in its absence may be relevant to the previous observation. Caveolae are hypothesized to be subcellular domains that regulate intracellular Ca^{2+} concentration and Ca^{2+}-dependent signal transduction [24]. The addressing of striatin to such caveolin-rich domains would possibly modulate the occupancy of their Ca^{2+}–calmodulin-binding domain and hence their subcellular localization.

Caveolins are known to interact with signalling proteins. The present study thus comforts our hypothesis concerning a possible signalling role for the proteins of the striatin family. Some tyrosine kinases, Ras, Gα, eNOS, interact with caveolin in their inactive form [11–13,17]. On the contrary, the binding of the androgen receptor to caveolin results in its enhanced activation [20]. Although the effects of striatin down-regulation are major [2], the molecular roles of the proteins of the striatin family are not yet known. However, besides caveolin, some of their partners are known: calmodulin and phocein. Phocein, a major partner of the proteins of the striatin family, is most probably involved in vesicular traffic [3]. As many cytosolic proteins are now known to play dual roles in endocytic processes as well as in signalling [25], it is likely that the proteins of the striatin family, bearing both a calmodulin-binding site and a WD-repeat, scaffolding domain, function in both processes.

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