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Monoclonal antibodies to α I spectrin Src homology 3 domain associate with macropinocytic vesicles in nonerythroid cells*

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Abstract

Spectrins represent a family of membrane-associated proteins responsible for membrane flexibility and cell shape in erythrocytes, and probably in most nonerythroid cells. Spectrin functions as a tetramer consisting of two heterodimers each containing two subunits termed α and β . In humans, α I and α II spectrins but not β spectrins are characterized by the presence of an Src homology 3 (SH3) domain. As a tool to investigate the function of spectrin SH3 domains we derived several monoclonal antibodies (mAb) to the recombinant human α I or α II spectrin SH3 domain. Immunostaining using these monoclonal antibodies indicated expression of α I spectrin in cell bodies and α II spectrin in neurites of granule neurons in mouse primary cerebellar cultures. Monoclonal antibodies reactive to α I spectrin SH3 domain indicated expression of a protein(s) containing an α I-like SH3 domain in cytoplasmic vesicular-like structures in GFAP-positive cells in these cultures. In NIH 3T3 fibroblasts, these antibodies label macropinocytic vesicles. Together, these data and Western blotting results suggest expression of at least three spectrin-SH3 domain antibody-reactive proteins.

Keywords

Spectrin; Src homology 3 domain; Endocytosis; Macropinosome

It is known that α I (or erythroid) spectrin plays a vital role in the shape and stability of erythrocyte membranes. A similar function has been ascribed to α II spectrin (also called fodrin or nonerythroid spectrin) in neurons, which is an isoform of spectrin that is expressed in most tissues (reviewed in Refs. [1,7]). In the last decade, spectrin isoforms associated with intracellular organelles have been identified suggesting that spectrins play a universal structural role in intracellular membranes (reviewed in Refs. [4,5,9]).

Spectrin consists of two polypeptide chains, α and β , which associate as heterodimers. These heterodimers, in turn, associate head-to-head to form spectrin tetramers, which are considered a functional unit of spectrin (reviewed in Ref. [26,27]). Currently, two α -spectrin

*Nomenclature for spectrin isoforms used in this paper is according to Winkelmann and Forget [29].

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genes are known, encoding α I- and α II-spectrin, respectively [13,18], and five genes encode β -spectrins [10,13–16,21,22,30]. Considering the $\alpha\beta$ heterodimer as a functional unit, the apparent imbalance in the number of α - vs. β -spectrins may be explained by hybrid heterodimer formation, e.g. α I with β II or β III, or α II with β I or β IV, as previously suggested [2,3]; or by the existence of additional undetected genes encoding other α -like spectrins that form functional heterodimers with β spectrins.

Apart from several 106-amino acid repeat units common to both α - and β -spectrins [20]; mammalian α -spectrins are uniquely identified by the presence of calcium binding sites (EF hands) and an Src homology 3 (SH3) domain. In both α I- and α II-spectrin, the SH3 domain is located in the mid-region of the molecule between repeat units 9 and 11 [13,18,31]. Although most binding properties of spectrin to other proteins have been localized in β spectrins (reviewed in Refs. [5,9]) the spectrin SH3 domain may function through interactions with cytoplasmic ligands, and we recently identified a candidate α I SH3 domain binding protein [33]. This protein, designated, Hsshb3p1, belongs to a family of tyrosine kinase-binding proteins [19,28,34]. The spectrin SH3 domain binding site is highly conserved in these proteins suggesting that spectrin may provide a scaffold for intracellular signaling proteins [33]. As a tool to investigate spectrin function, we produced and characterized antibodies that detect SH3 domains from different isoforms of α -spectrin. Immunostaining and Western blotting analysis using these antibodies suggest expression of a protein(s) containing an α I-spectrin-like SH3 domain which associates with endocytic compartments in many nonerythroid cells, including GFAP-positive cells in mouse primary cerebellar cultures.

Purified GST fusion proteins containing the human α I- SH3 domain (GST-E-SH3) or the human α II-SH3 domain (GST-F-SH3) [33] were used for immunization of mice. Monoclonal antibodies were derived at the Institute for Basic Research in Developmental Disabilities Antibody Facility using standard techniques. Reactivities of antibodies to the recombinant spectrin SH3 domains were evaluated by ELISA and Western blotting. All antibodies reactive with GST and not to either of the spectrin SH3 domains were omitted from further analysis. Western blotting was performed using a PVDF membrane as described [11]. Polypeptides were separated on 7% SDS–Tricine polyacrylamide gels (GST fusion proteins), or on low-bis 6% SDS–Tris polyacrylamide gels [6] (NIH 3T3 cell lysates).

Cerebellar cell cultures were prepared as described [25,26]. Briefly, whole brains were removed from postnatal day-7 (P7) mouse pups (C57BL/6) and cell dissociation from cerebella was accomplished by trituration with a series of fire-polished Pasteur pipettes. After centrifugation cell pellets were resuspended in serum supplemented culture medium (10% horse serum; 5% FCS; 0.25% glucose (w/v); penicillin, 50 U/ml; streptomycin, 50 μ g/ml, in MEM). Cells were seeded onto poly-D-lysine-coated (100 μ g/ml) coverslips at a density of 1.875×10^6 cells/ml, and incubated at 37°C, in 5% CO₂. After 24 h, the culture medium was changed to serum free medium (0.25% glucose (w/v); penicillin, 50 U/ml; streptomycin (50 μ g/ml); 0.1% N2 supplement (Life Technologies, Rockville, MD)), in MEM. Cells were incubated an additional 48 h prior to processing for immunofluorescence. Cells were fixed in 100% methanol for 10 min at room temperature (cerebellar cultures) or with ice cold methanol (cultured cell lines). NIH 3T3, MDCK, HeLa, PTK1, and NRK cells

were obtained from and grown according to ATCC (Rockville, MD) instructions. Tissue culture media were from Life Technologies or from Fisher Scientific (Pittsburgh, PA).

Uptake of the fluorescent markers wheat germ agglutinin–texas red conjugate (WGA-TR) (at 10 $\mu\text{g}/\text{ml}$) and Alexa 594 (1 mg/ml) (Molecular Probes, Eugene, OR) by NIH 3T3 cells was monitored at several time points (from 5 min up to 2 h after addition of the marker to medium) following fixation of cells. MAbs to spectrin SH3 domain were used as primary antibodies in Western blotting and in indirect immunofluorescence. Secondary antibodies were from Jackson ImmunoResearch, Inc. (West Grove, PA) and from Molecular Probes. Coverslips were rinsed extensively with PBS and mounted on ethanol-cleaned glass slides with mounting media as described [33]. High-resolution images (1024 \times 1024 pixels) were collected using a 60 \times NA 1.4 objective and a Nikon PCM 2000 Confocal Imaging System.

Out of 17 mAb clones, reactive with spectrin SH3 domain but not with GST, three groups of clones were defined based on their immunoreactivity in Western blotting (Fig. 1). One group of clones, represented by mAb 13C2, reacted with both αI and αII spectrin SH3 domains. While mAb 15A5 is also in this group, it showed less reactivity with the αII SH3 domain than did mAb 13C2. The second group of clones, represented by mAb 13C5 and mAb 17C7, reacted specifically with the αI SH3 domain. The third group of clones, represented by mAb D8B7, reacted specifically with the αII SH3 domain. Immunoreactivity of these mAbs in ELISA (not shown) was very similar to that in Western blot analysis. All antibodies except mAb D8B7 reacted with αI spectrin (240-kDa band) extracted from human ghosts (Table 1). MAbs 13C2 and 15A5 reacted with the 240-kDa species in lysates of NIH 3T3 cells. The same reactivity was observed with mAb D8B7 in NIH 3T3 cells (Fig. 2). MAb 17C7 reacted with αI spectrin in mouse ghosts and with a 200-kDa protein in NIH 3T3 cells and in mouse cerebellum [35]. MAb 17C7 showed weak reactivity with a 180-kDa band in NIH 3T3 cells, while mAb 13C2 showed reactivity with a 200-kDa band and a 180-kDa band in these cells (Fig. 2).

Immunoreactivity of spectrin SH3 domain antibodies was evaluated in primary mouse cerebellar cultures. MAb D8B7 labeled neurites and the periphery of cell bodies in granule neurons (Fig. 3a). In cerebellar neurons mAb D8B7 staining colocalized with that of polyclonal antibody 992 raised to axonal spectrin (Fig. 3b) [17]. MAbs 15A5, 13C5, and 13C2 showed immunoreactivity with granule neuron cell bodies (Fig. 3c). MAb 17C7 as well as the other antibodies reactive with the αI SH3 domain (mAbs 13C2, 13C5, and 15A5) showed a cytoplasmic punctate immunostaining pattern in GFAP-positive cells (Fig. 3e and f) and in fibroblastic-like cells present in primary cerebellar cultures. Furthermore, these antibodies stained cytoplasmic vesicular-like structures in mouse NIH 3T3 fibroblasts (Fig. 3d) and in HeLa, MDCK and NRK, and PTK1 cells (not shown). MAb 13C2 (Fig. 4) as well as other mAbs to the αI spectrin SH3 domain colocalized with vesicles containing fluorescent WGA-TR endocytosed by cells within 5 min. The functional properties of these vesicles are consistent with macropinosomes [35]. Thus, mAbs to the αI SH3 domain serve as markers of macropinosomes.

The SH3 domain is highly conserved in spectrins. Nonerythroid spectrins from *Drosophila*, mouse, chicken, rat and human all contain an SH3 domain [24]. Sequences of the SH3

domain in human α II-spectrin and chicken α -spectrin are 100% identical [13,31]. α I- and α II-spectrins are the most homologous in the SH3 domain region, with 65% identity compared to an overall identity between these spectrins of 54%. The specificity of mAb D8B7 for the α II spectrin SH3 domain was consistent with expression of α II spectrin in neurites of granule neurons and the plasma membrane of nonerythroid cells [12,17]. MABs reactive with the α I SH3 domain indicated expression of α I spectrin in cell bodies of granule neurons consistent with observation by Clark et al. [2]. Interestingly, all mAbs reactive with the α I SH3 domain associated with macropinocytic vesicles in NIH 3T3 cells as defined by Xu et al. [35]. The macropinocytic compartment was originally characterized in macrophages and in tumor cell lines (reviewed in Ref. [23]). Macropinocytic vesicles are resistant to brefeldin A treatment, do not contain a clathrin coat, are heterogeneous in size, and are larger than clathrin-coated vesicles [8,32]. The similar staining pattern of GFAP-positive cells in cerebellar primary cultures and in many nonerythroid cultured cell lines suggest the presence of macropinocytic vesicles in astrocytes and in many cells originating from different tissues. Our antibodies provide useful markers in further defining the function of this non-clathrin pinocytic compartment.

The staining of macropinocytic vesicles with anti- α I SH3 domain antibodies suggests expression of proteins containing an SH3 domain similar to the α I SH3 domain. A 200-kDa protein that reacts with mAb 17C7 and is a Hsshb3p1 interacting protein [35] is also detected by mAb 13C2, which detects an additional 180-kDa band. The lack of mAb 13C5 reactivity with these bands, and the labeling of macropinocytic vesicles by this antibody suggest the presence of a conformational epitope shared by the α I spectrin SH3 domain, the 200-kDa protein and the 180-kDa band. Our mAbs will help to determine whether these bands represent novel α -spectrins.

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Abbreviations

ATCC	American Tissue Culture Collection
BSA	bovine serum albumin
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GFAP	glial fibrillary acidic protein
GST	glutathione S-transferase
MEM	minimal essential medium
mAb	monoclonal antibody

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidene difluoride
SH3	Src homology 3
Tricine	<i>N</i> -[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine

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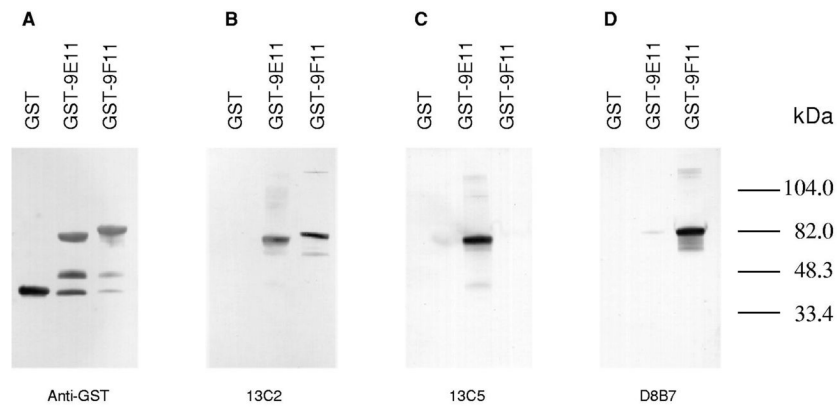


Fig. 1.

Specificity of monoclonal antibodies to recombinant spectrin Src homology 3 domain. Equal amounts of IPTG-induced bacterial lysates of the GST-SH3 domain fusion polypeptides of α I spectrin (GST-9E11, containing spectrin repeat units 9 through 11, as defined in Ref. [11]) and α II spectrin (GST-9F11, containing α II spectrin repeat units 9 through 11; it includes two sequence insertions not present in α I spectrin and accounting for an apparent difference in migration between GST-9E11 and GST-9F11) were solubilized in Laemmli buffer and separated on 7% SDS-Tricine polyacrylamide gels. Identical blotted transfers of gels were incubated with the following antibodies: anti-GST, panel A; mAb 13C2, panel B; mAb 13C5, panel C, and mAb D8B7, panel D. mAb 17C7 showed reactivity identical to mAb 13C5; mAb 15A5 was similar to mAb 13C2 but was less immunoreactive to GST-9F11. All monoclonal antibodies were raised to recombinant GST fusion polypeptides as indicated in Table 1.

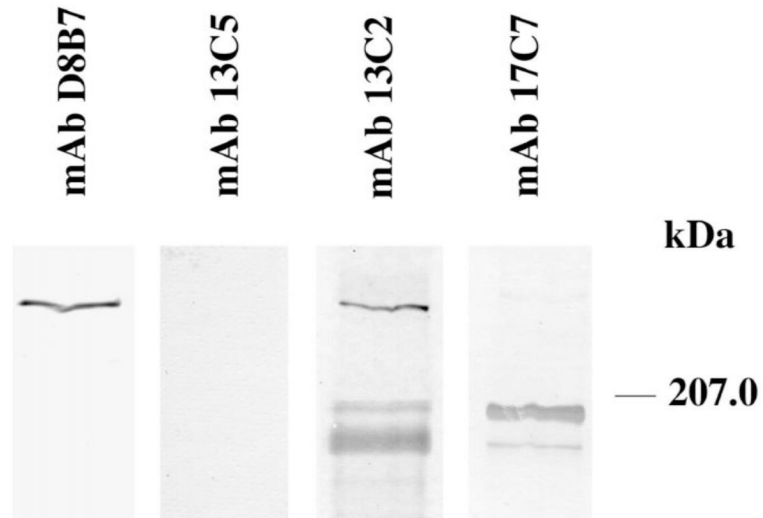


Fig. 2. Reactivity of antibodies in NIH 3T3 cells. Samples containing 20 μ g of total cell lysate were separated on 6% low-bis SDS–Tris poly-acrylamide gels. Identical blotted transfers of gels were incubated with the antibodies indicated above panels. MAb 15A5 reactivity (not shown) was similar to mAb D8B7.

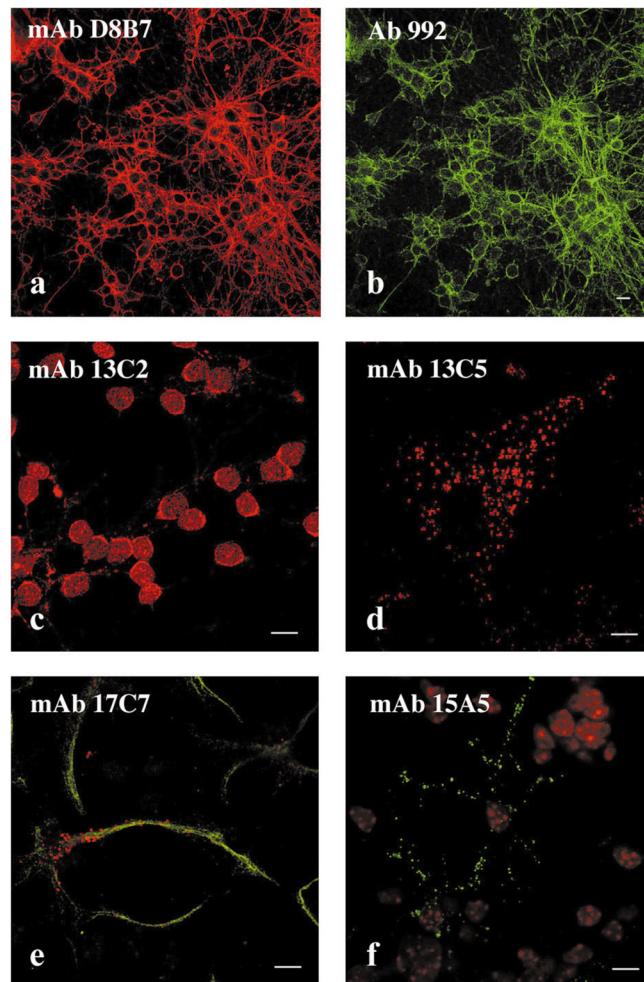


Fig. 3. Immunostaining pattern of monoclonal antibodies to spectrin SH3 domains. Mouse cerebellar cultures (a–c and e and f) and NIH 3T3 cells (d) were stained with monoclonal antibodies to spectrin SH3 domain. A culture enriched for granule neurons costained with mAb D8B7 to α II SH3 domain (a, red) and with the polyclonal antibody 992 to axonal spectrin [17] (Chemicon, Temecula, CA) (b, green), or stained with mAb 13C2 (c) (red). A culture of cerebellar cells enriched for astrocytic cells costained with mAb 17C7 (e, red) and with the polyclonal antibody to GFAP (Sigma, St. Louis, MO) (f, green). An NIH 3T3 fibroblast stained with mAb 15A5 (d) (green), nuclear autofluorescence (red). Antibodies 17C7, 13C2, and 15A5 produce very similar pattern in NIH 3T3 cells to that observed with mAb 13C5. Note coincident pattern in (a) and (b), indicating colocalization of antibody staining. Bar, 10 μ m.

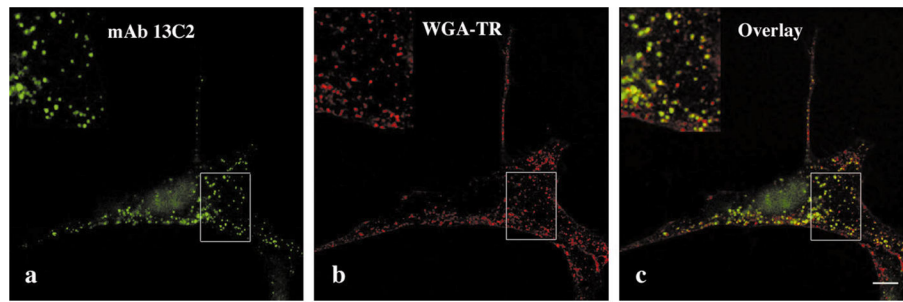


Fig. 4. MAb 13C2 to α I spectrin SH3 domain labels vesicles participating in endocytosis of fluorescent wheat germ agglutinin in NIH 3T3 cells. (a–c) Cells were allowed to endocytose WGA-TR (red) for 5 min and stained with mAb 13C2 (green); (c) is overlay of (a) and (b) images. Similar result was observed with all monoclonal antibodies reactive to the α I SH3 domain. Bar, 10 μ m.

Table 1

Characterization of monoclonal antibodies to α I and α II spectrin SH3 domains

MAb clone	Antigen ^a	Immunoreactivity in Western blotting			Immunofluorescence in cultured cells			
		E-SH3	F-SH3	Human ghosts ^b	NIH 3T3 cells ^c	NIH 3T3 cells	Primary cerebellar culture	Glial cells ^d
D8B7	F-SH3	-	+++	-	240 kDa	Cell periphery	Cell extensions and periphery	Cell periphery (weak)
13C2	E-SH3	++	++	240 kDa	240 kDa 200 kDa ^e 180 kDa			
13C5	E-SH3	+++	-	240 kDa	-	Punctate cytoplasmic	Cell bodies	Punctate cytoplasmic
15A5	E-SH3	+++	+/-	240 kDa	240 kDa			
17C7 ^f	E-SH3	+++	-	240 kDa	200-kDa 180 kDa ^e		-	

^a Recombinant GST-SH3 polypeptides containing α I spectrin (E-SH3) and α II spectrin (F-SH3) SH3 domains [33] were used as antigens. '-', indicates no reactivity; '+/-' weak reactivity; '++' moderate reactivity; '+++' strong reactivity.

^b α I spectrin was extracted from human ghosts as described in Ref. [3].

^c Total cell lysate of NIH 3T3 cells was used. Apparent molecular size of immunoreactive bands is indicated (see also Fig. 2).

^d GFAP-positive cells.

^e Weak reactivity.

^f MAb 17C7 was previously reported in Ref. [35]. Weak reactivity to the 180-kDa is observed.