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A START-Domain-containing Protein is a Novel Marker of Nervous System Components of the Sea Cucumber *Holothuria glaberrima*

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Abstract

One of the main challenges faced by investigators studying the nervous system of members of the phylum Echinodermata is the lack of markers to identify nerve cells and plexi. Previous studies have utilized an antibody, RN1, that labels most of the nervous system structures of the sea cucumber *Holothuria glaberrima* and other echinoderms. However, the antigen recognized by RN1 remained unknown. In the present work, the antigen has been characterized by immunoprecipitation, tandem mass spectrometry, and cDNA cloning. The RN1 antigen contains a START lipid-binding domain found in Steroidogenic Acute Regulatory (StAR) proteins and other lipid-binding proteins. Phylogenetic tree assembly showed that the START domain is highly conserved among echinoderms. We have named this antigen HgSTARD10 for its high sequence similarity to the vertebrate orthologs. Gene and protein expression analyses revealed an abundance of HgSTARD10 in most *H. glaberrima* tissues including radial nerve, intestine, muscle, esophagus, mesentery, hemal system, gonads and respiratory tree. Molecular cloning of HgSTARD10, consequent protein expression and polyclonal antibody production revealed the STARD10

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Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contributions

EAOR performed or participated in most of the experiments in this study. EAOR and JEGA conceived the study, participated in its design and coordination and wrote the manuscript. JHP, GARO, CADB and GVT were involved in the production of the STARD10 antibody and the immunohistological results. GARO participated in the cloning and expression of the STARD10 gene and the purification of the protein. EVR and IEV were in charge of the Mass Spectrometry experiments and the corresponding data analyses. All authors read and approved the final manuscript.

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ortholog as the antigen recognized by the RN1 antibody. Further characterization into this START domain-containing protein will provide important insights for the biochemistry, physiology and evolution of deuterostomes.

Introduction

Echinoderms, being invertebrate deuterostomes, may provide important insights into the evolution of the nervous system organization of chordates. One of the peculiarities of the echinoderm nervous system is its adult radial symmetry that differs from the bilateral symmetry of other deuterostome animals (Hyman 1955, Mashanov et al. 2015). The echinoderm central nervous system is composed of an anterior nerve ring connected to radial nerve cords that extend to the posterior end of the animals. Both the nerve ring and the radial nerve cords are ganglionated structures, where neuronal cell bodies are found in the periphery of the structure while nerve fibers compose most of the central part. In the members of the class Holothuroidea (sea cucumbers) the radial nerve cords is divided into two components: the ectoneural component thought to have both sensory and motor functions, and the hyponeural component, that mainly motor functions, innervating the principal muscle systems of the organisms. Echinoderms, like other animals, have a peripheral nervous system component that has been well characterized in some organs such the digestive tract (Garcia-Arraras et al. 2001) and the tube feet (Diaz-Balzac et al. 2010).

One impediment to the study of the echinoderm nervous system has been the lack of markers that may describe the development, function and anatomy of their nervous system. This lack of markers has been an ongoing limitation in our research on nervous system regeneration where we use an echinoderm model system, the sea cucumber *Holothuria glaberrima* (San Miguel-Ruiz et al. 2009, Mashanov et al. 2013, Tossas et al. 2014). To tackle this problem, the search and characterization of new markers capable of identifying neurons and plexi that form the echinoderm nervous system components are needed. Our lab and those of others have identified various antibody markers for cells and fibers (Diaz-Miranda et al. 1995a,b, 1996, Diaz-Balzac et al. 2010, 2012, 2014, 2016). In general, the markers fall into two large categories; the first being those antibodies that recognize what are supposed to be the echinoderm homologs of the antigens recognized by the antibodies in other animal species. Here we find antibodies to neurotransmitters such as serotonin (Murabe et al. 2008), GABA (Newman and Thorndyke 1994) and histamine (Hoekstra et al. 2012) or to their synthesizing enzymes, such as tyrosine hydroxylase (Diaz-Balzac et al. 2010). Other antibodies recognize echinoderm peptides (Diaz-Miranda et al. 1995), or proteins associated with the nervous system such as synaptotagmin (Burke et al. 2006), calbindin (Diaz-Balzac et al. 2012) or β -tubulin (Diaz-Balzac et al. 2016). The second category are antibodies that label specific components of the nervous system, but whose target antigen is not known. Antibodies in this category include an antibody made against phosphorylated histone but whose labeling in the holothurian nervous system suggests cross labeling of a different molecule found within a specific neuronal population (Diaz-Balzac et al. 2014) and an antibody that recognizes holothurian glial cells (Mashanov et al. 2010).

One of the markers in the second group is a monoclonal antibody, RN1, developed in our laboratory that labels most of the nervous components of the nervous system of sea cucumbers or holothurians, members of the Class Holothuroidea within the Echinodermata phylum (Diaz-Balzac, 2007). In the sea cucumber *Holothuria glaberrima*, RN1 labels all previously described nervous structures including the ectoneural and hyponeural divisions plus many previously undescribed components such as large fiber plexi and cells within many organs and tissues (Diaz-Balzac et al. 2007). These nervous structures also show positive immunostaining to neuropeptides and other markers of neural components (Diaz-Balzac, 2007, 2014).

Although the RN1 antibody has served its purpose as a marker of holothurian nervous system components, the lingering question remains as to what is the molecule it recognizes. The answer to this question will not only provide information on the circuitry and anatomy of the nervous system but will also help identify what appears to be a protein that is ubiquitous in different areas of the nervous system, suggesting that it may play an important role in neural function. Here, we describe the characterization of RN1 antigen by immunoprecipitation and tandem mass spectrometry, as being a START-domain-containing protein. We also demonstrate the expression of the identified protein and gene transcript levels in different tissues and identify other members of the START domain family present in echinoderms.

Experimental Procedures

Animals

Adult sea cucumbers (*H. glaberrima*) specimens were collected in the surrounding waters of Puerto Rico and maintained in seawater aquaria at 22–24°C with circulating seawater and constant oxygenation.

Preparation of tissue extracts

Sea cucumber tissues including the radial nerve complex, intestine, mesentery, respiratory tree, muscle, hemal system and gonads were dissected and homogenized using Polytron in Tri Reagent Extraction Solution (Molecular Research Center, Inc., Cincinnati, OH) followed by protein and RNA extraction using the standard phenol/chloroform extraction recommended by supplier. Protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL).

RN1 immunoprecipitation and Mass Spectrometry Analysis

Animals were anesthetized by placement in ice-cold water for 1 hour. The radial nerve complex of animals, which includes the nerve cord and part of the body wall muscles, was dissected. Samples were maintained in 1 mL of RIPA buffer with Complete Protease Inhibitors cocktail (Santa Cruz Biotechnology) and homogenized in this solution using a Polytron (Brinkmann Instruments). The homogenate was centrifuged (10,600 ×g, 4°C for 10 minutes) and the supernatant was transferred to another tube. The supernatant was incubated with 2 µL of undiluted ascites fluid of RN1 antibody, continuously moving for 1 hour at 4°C. As a control, 20 µL of Protein A/G PLUS agarose beads (Santa Cruz Biotechnology)

was added to another sample, incubated in the same conditions, centrifuged (10,600 ×g, 4° C for 10 minutes), and the pellet was stored at –20°C for western blot analysis. After incubation with the antibody, 20 µL of agarose beads were added to the sample and incubated overnight (continuously moving, 4°C). Next morning, the sample was centrifuged for 5 minutes (2,500 ×g 4°C) and the supernatant was discarded. Samples were washed with 1 mL of RIPA buffer and centrifuged for 5 mins (2,500×g 4°C). This step was repeated twice, and the supernatant of the last centrifugation was used for western blot analysis. The pellet after the last centrifugation corresponds to a mixture of immunoprecipitated molecules with the agarose beads.

Immunoprecipitated samples were run at 200 V for 50 minutes in 12% SDS-PAGE under denaturing conditions. The gel was stained with Coomassie brilliant blue (Thermo Fisher Scientific, Waltham, MA) and both bands were manually excised and processed independently. Excised bands were destained and digested using 1µg of trypsin for 18 hours at 37°C. HPLC with a PicoFrit™ column packed with ProteoPep™ C18 (New Objective, Woburn, MA) was used to separate the tryptic-peptides and eluted using a linear gradient (55 min) of water:acetonitrile (20%:80%) in 0.2% formic acid. The samples were analyzed in an LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Identification was performed using Thermo Proteome Discoverer version 1.2.0.208 (Thermo Fisher Scientific, Waltham, MA) that correlates MS–MS spectra with sequences from translated cDNA libraries of radial nerve and regenerating intestine databases using SEQUEST database search algorithms (Ortiz-Pineda et al. 2009, Mashanov et al. 2014, Mashanov et al. 2015). All the sequences from these libraries were translated using the EMBOSS sixpack algorithm. The search parameters used were: two trypsin missed cleavage sites allowed, the precursor and fragment tolerance was set to 2 Da and 1 Da respectively. The decoy database search was set to target false discovery rate strict and relaxed to 0.01 and 0.05, respectively. Only peptides with Xcorr higher than 1.5 (+1), 2.0 (+2), or 2.25 (+3) and high confidence were considered in the study.

Bioinformatics analysis of sequences

Sequenced samples were assembled and analyzed using Geneious Pro 4.7.6. Multiple sequence alignment of the sea cucumber's STARD10 homologous protein sequence and proteins of evolutionarily close organisms that showed the highest similarity in NCBI BLASTX including the sea star *P. pectinifera* (ID: BAF98202.1) and the sea urchin *S. purpuratus* (ID: XP_790235.3) was performed using the MUSCLE algorithm. The alignment was computed using the following parameters: Kmer4-6 distance measure for the initial iteration and kimura percentage identity for subsequent iterations, gap open score of –1, anchor spacing of 32, hydrophobic window size of 5, and diagonals of minimum length 24 with a margin of 5, and minimum anchor score of 5 (Edgar, 2004). All the START domain-containing protein sequences from humans and other model organisms including the nematode *C. elegans*, the fruit fly *D. melanogaster*, and the sea urchin *S. purpuratus* were obtained from GenBank (NCBI; Supplementary Table 1). Each human START domain-containing protein was queried against the cDNA libraries of regenerating intestine and radial nerve tissue from our group using BLAST algorithm. The evolutionary relationship among all the START domain protein sequences from the four organisms, including the

three predicted for our model organism, was determined through a phylogenetic tree analysis using the UPGMA tree building method (Nei et al., 2000) and the genetic distances and probabilities calculated using the Jukes-Cantor model (Jukes and Cantor, 1969).

Western Blots

Samples of equal protein concentrations from tissue homogenates, immunoprecipitated samples, immunoprecipitation controls and bacterial homogenates were run at 200 V for 50 minutes in 12% SDS-PAGE under denaturing conditions. The gel was equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for 15 min. The transfer was performed at 100 V for 1 hour on ice in a Mini Trans Blot Cell. The PVDF membrane was incubated for 1 hour in 5% nonfat dry milk as a blocking solution, washed three times (Tris-buffered saline with 0.2% Tween 20), and incubated overnight in RN1 antibody diluted either 1:5,000 (tissue expression analysis and recombinant protein specificity) or 1:10,000 (immunoprecipitation experiments) with RPMI 1640 medium supplemented with 5% horse serum. Similar protocols were followed with anti-HgSTARD10 antibodies (1:300) in the sera of mice immunized with the recombinant protein. After three washes of 20 min each, the membrane was incubated for 1 hour in secondary antibody (sheep anti-mouse IgG peroxidase-linked Cell Signaling Technology®) diluted 1:5,000 with the same RPMI 1640 supplemented medium. After three more washes of 20 min each, the membrane was incubated with Super Signal West DURA Chemiluminescent detection reagents (Thermo Fisher Scientific, Rockford, IL) for 5 minutes and visualized in a ChemiDoc XRS+ System (BioRad Molecular Imager GelDoc™ XR). For the loading control, the PVDF membrane was incubated overnight in beta-tubulin antibody (Thermo Fisher Scientific # MA5-16308) diluted 1:5,000 overnight and the same secondary antibody was used following the procedure described above.

Semi quantitative-PCR (sqPCR)

Primers were designed for optimal performance using Geneious Pro 4.7.6, utilizing a contig with a domain homologous to START. PCRs were performed in the Mastercycle (Eppendorf) using the Promega's Taq polymerase kit. To detect tissue expression of the mRNA, we used the following set of primers: RNIF F5'-TGTCTCAGTCGTGTGCGCCG-3' and RN1R2 R5'-GCCGCCGGGGTCTCTTCAAC-3'. Equal concentrations of cDNAs from multiple tissues including the radial nerve, intestine, muscle, esophagus, mesentery, hemal system, gonads and respiratory tree of 3 animals were used as a template for amplification. Cycling conditions for the amplified products were as follow: 94°C × 1 min, 64.6°C × 1 min, 72°C × 1 min; all performed for 26 cycles. Samples of the amplified products (~7 µL) were analyzed by electrophoresis in a 1 % agarose gel to determine the size of a PCR product for both primer pairs. DNA quantitation by gel densitometry was performed to obtain relative expression values of HgSTARD10 in different tissues. DNA samples were sequenced at the Sequencing and Genotyping facility at UPR-Rio Piedras.

Cloning of *H. glaberrima* STARD10

cDNA was prepared from RNA isolated from radial nerve tissue extractions (see Mashanov et al. 2014, 2015). Using this template, we amplified the START domain gene with the

primer pair RN1F and RN1R2. This product was purified using the ExoSap (USB) protocol. The PCR product was cloned into the pET 200 TOPO vector for 15 mins at room temperature following the protocol of Champion pET 200 TOPO Expression kit (Invitrogen). Afterwards, the construct was heat-shocked into OneShot TOP10 chemically competent *E. coli* cells (Invitrogen) and incubated in SOC medium (Invitrogen) at 37° C for 1 hour before plating. We screened for positive insertion using selective plating with ampicillin and Colony PCR, using specific primers supplied by the Champion pET 200 TOPO Expression kit (Invitrogen). All constructs were analyzed in 1% agarose gels run at 120 V for 40 minutes in 10 mM Sodium Borate buffer, visualized with SafeView Classic (Applied Biological Materials) staining in a ChemiDoc XRS+ System. Sanger Sequencing verified bacterial colonies that were positive for STARD10 insertion.

Recombinant STARD10 expression and antibody production

Positive bacterial colonies were grown in LB medium with 50 mg/ml ampicillin to O.D.600 0.7, and induced with 1 mM IPTG (Sigma, St. Louis, MO). Cells were lysed using B-PER Reagent (Thermo Fisher Scientific, Rockford, IL) and protein extraction was carried out using TRIzol LS Reagent (Invitrogen, Calsbad, CA) according to the manufacturers protocol for bacterial protein extraction. The fractions were then analyzed via SDS-PAGE and western blot. For antibody production, recombinant STARD10 in protein suspension from B-PER extraction was purified using a Ni-NTA purification system (Thermo Fisher Scientific, Rockford, IL), and fractions were collected after lysing, washing, and elution following the company's protocol. As a positive control, another protein from the same bacterial strain containing the same vector but a different insert, ORPIN, was purified in parallel with our recombinant protein. Approximately 50 µg of purified protein was diluted with PBS and Freund's Complete Adjuvant (Sigma, St. Louis, MO) in a 1:1 solution to a final volume of 0.3 mL and injected intraperitoneally into 2 mice. Another injection was performed three weeks after. A week after the second injection, blood was collected from retro-orbital plexus bleeding and incubated with 1mM PBS at 37°C for 1 hour to retrieve sera. Samples were centrifuged at 12,000×g for 5 mins and serum was collected and stored at 4°C. The supernatant was subsequently used for immunohistochemistry and western blots. All animal experiments were approved by the IACUC of UPR.

STARD10 detection by immunohistochemistry

Immunohistochemistry was used to compare the expression of STARD10 and RN1 as described before (Diaz-Balzac et al. 2007). RN1 ascites fluid was used at a 1/300,000 concentration, while STARD10 antiserum was used at 1/200.

Results

RN1 antigen identification using mass spectrometry

To elucidate the identity of the RN1 antigen, we combined immunoprecipitation of radial nerve protein homogenates and mass spectrometry. Immunoprecipitation experiments with the RN1 antibody were performed successfully (Figure 1a). In brief, radial nerve homogenates were incubated with the antibody, and the antigen-antibody complex was immunoprecipitated with protein A-G agarose beads. Western blot analysis of the IP

complex showed the expected size bands for the RN1 antigen of 53 and 66 kDa. Binding of the antigen was specific to the antibody/agarose bead complex as we did not observe its enrichment in homogenates incubated with protein A-G agarose beads only (Fig. A, BA). After demonstrating that RN1 can immunoprecipitate proteins, the proteins obtained from the IP were resolved by SDS-PAGE, stained and the detected bands corresponding to the 53 and 66 kDa species were digested with trypsin for protein identification using tandem mass spectrometry analysis. The spectra obtained were mapped onto the sequences of translated cDNA libraries of the sea cucumber's intestinal and radial nerve tissues (Ortiz-Pineda et al. 2009, Mashanov et al. 2014, Mashanov et al. 2015). At least 6 peptides were identified by MS/MS with a high confidence level and identified in the spectra from both the 53 and 66 kDa fractions (Figure 1b). All the peptides identified mapped onto the protein sequence deduced from a contig identified in the cDNA libraries. Conceptual translation of the contig identified an open reading frame (ORF) with an expected size of 52 kDa, a molecular weight similar to that of one of the species identified by western blot (Figure 1a). The detection of the two species that differ in molecular weight may suggest that they correspond to alternatively-spliced isoforms. However, we were not able to detect additional contigs that encode for ORFs with an expected size of around 66 kDa. Interestingly, chromatogram analysis also revealed that three peptides contained posttranslational modifications (Supplementary Figure 1). Combinations of 5 putative phosphorylation sites at Y135, S136, T140, Y147 and T148 were identified in at least 4 different peptides. Interestingly, the putative phosphorylated peptide was only identified in tryptic peptides isolated from the 66 kDa fraction.

Sequence conservation and phylogenetic analysis

In order to show that the contig assembled in our database and identified by mass spectrometry analysis corresponded to a holothurian mRNA transcript, primers were designed and used for PCR amplification of cDNA from the radial nerve. The obtained band matched the expected size of the contig and the product sequence corresponded to that of the assembled contig. It codified for a START domain-containing protein with high similarity to the STARD10 of the sea urchin *S. purpuratus* and the starfish *A. pectinifera* according to BLAST algorithm (Marchler-Bauer et al. 2013). Multiple sequence alignment analysis by MUSCLE algorithm showed high degree of conservation among echinoderms that include the sea cucumber *H. glaberrima*, the starfish *A. pectinifera*, and the sea urchin *S. purpuratus* (Figure 2). The alignment showed a significant 52.4% pairwise identity and high conservation in the predicted START domain that spanned approximately 205 residues of the putative protein.

START domain-containing protein sequences of *D. melanogaster*, *H. sapiens*, *S. purpuratus*, and *C. elegans* were obtained from NCBI for phylogenetic analysis based on sequence similarity (Supplementary Table 1). Holothurian orthologs were identified in our cDNA database using the sea urchin's mRNA sequences based on nucleic acid sequence conservation. Three different START domain-containing contigs were identified in the holothurian database. These included the previously identified contig with high similarity to STARD10 and two other putative START domain-containing proteins. Hierarchical clustering analysis showed that STARD10 groups with one ortholog of the human, and two

of the sea urchin including STARD2 and STARD10 (Figure 3). The other two holothurian orthologs clustered with STARD7 and STARD13 of the other species. Thus, we have named the three holothurian gene sequences HgSTARD10, HgSTARD7 and HgSTARD13 based on the sequence similarity and conservation. Two sea cucumber and two sea urchin sequences are members of the STARD2/PCTP subfamily and clustered together with others that included STARD2, STARD7, STARD10 and STARD11. An additional sequence of *H. glaberrima* together with one of the sea urchin grouped with members of subfamily 4 (STARD8, 12 and 13). Sea urchin sequences were found for subfamilies 1 (STARD1, 2 and 3) and 2 (STARD4, 5 and 6). None of the echinoderm sequences grouped with mammalian members of subfamily 5 (STARD14 and 15). Members of the subfamily 6 (STARD9) were not grouped together. Human STARD9 grouped with sequences of subfamily 1 while sea urchin's STARD9 grouped with sequences of subfamily 3.

Tissue specific expression

We analyzed HgSTARD10 expression at both the gene transcript and protein level in different tissues. Since the protein is found along nerve fibers that might lie distant from the cell bodies, the Western blot values should provide a better correlation with what has been observed in immunohistochemical studies. Nonetheless, we also performed sqPCR to provide a broad view comparison of the expression of the mRNA. It was decided that qRT-PCR would not provide major information that could not be obtained with the sqPCR. Various tissues were dissected including radial nerve, intestine, esophagus, muscle, respiratory tree, hemal system and gonads for protein and RNA extractions. HgSTARD10 is expressed in most tissues with the highest expression in the radial nerve as assayed by sqPCR (Figure 4). Correlating with our gene expression data, there is a high abundance of HgSTARD10 protein in the radial nerve but there is also low to mid-levels of protein expression in some of the tissues assayed including the hemal system, muscle, gonads, esophagus and mesentery. Relative differences in expression among tissues were not directly assessed as there is significant variation in the expression of normalizer genes in the tissues included in this analysis (Figure 4c). Thus, estimation of protein expression was only carried out by normalizing to total protein concentration loaded. However, our results are in agreement with previous characterization of RN1 immunoreactivity in the connective tissue and nerve plexi in the tissues included in this report (Díaz-Balzac et al., 2007).

Cloning and expression of STARD10 for antigen validation

To confirm that the antigen recognized by the RN1 antibody is indeed HgSTARD10, we cloned the ORF, contained within the contig identified by MS, into a bacterial expression vector and purified the recombinant protein. The RN1 antibody only recognized recombinant HgSTARD10 protein with a weak immunoreaction whereas an antibody specific to the poly-histidine tag residues located in the N-terminal of our recombinant HgSTARD10 protein recognized a band with a molecular weight around 53 kDa (Supplementary Fig. 2). This validates that the recombinant protein is being expressed but questions the specificity of the RN1 antibody. One possible explanation for the poor recognition of RN1 is that the antibody recognizes a post-translational modification in HgSTARD10 (i.e. phosphorylated residues) and that these post-translational modifications do not take place when the protein is expressed in bacteria. Thus, to further confirm the

identity of RN1 antigen, an antiserum against purified recombinant HgSTARD10 was developed to compare its labeling pattern against RN1 labeling in *H. glaberrima* tissues.

Characterization of antibodies against HgSTARD10

Two mice were immunized with purified recombinant protein. Sera obtained following the second injection (first booster) showed labeling of the radial nerve, the main nervous structure of holothurians in immunohistochemical analyses. The anti-HgSTARD10 polyclonal antibodies labeling is virtually identical to that of the monoclonal RN1 antibody; no obvious differences could be found when RN1 labeling was compared to that of the anti-HgSTARD10 antibodies (Fig. 5a–d). Both labeled the ectoneural and hyponeural subdivision, with a strong labeling of the central neuropile and a weak labeling of the cell bodies found in the nerve borders. Similarly, both labeled the nervous plexi in the body wall (Fig. 5a–d), the enteric nervous system, muscle and mesentery (data not shown). The HgSTARD10 antisera were also tested in Western blots of radial nerve and muscle protein extracts. Sera from both mice recognized the expected bands at 53 and 66 kDa that are recognized by the RN1 antibody, but with differential affinity (Figure 5e). It is apparent that the intensity of the 66 kDa band is lower than the 53 kDa in western blots with the mouse antisera contrasting with the staining pattern of the immunoprecipitated RN1 antigen. This result suggests that the RN1 antibody binds with higher affinity to a putative protein isoform in native versus denaturing conditions (Diaz-Balzac, 2007). Regardless of the difference in intensity, both RN1 and mouse HgSTARD10 antisera detect similar proteins bands in western blot analyses. Thus, the results validate HgSTARD10 as the antigen recognized by the nerve-specific RN1 antibody.

Discussion

HgSTARD10 is the antigen recognized by the RN1 antibody

Our results demonstrated that the START-domain-containing protein HgSTARD10 is the antigen recognized by the RN1 antibody, a marker of diverse nervous system structures in *H. glaberrima*. First, mass spectrometry analysis revealed an abundance of this protein in RN1 immunopurified samples. At least 6 peptides identified mapped onto the predicted protein sequence from a contig of our cDNA libraries with significant correlations based on values obtained from protein identification algorithms (Eng et al. 1994). Second, the putative contig was identified and characterized from cDNA obtained from radial nerve complex tissue. Third, there is good concordance in the expression of the HgSTARD10 transcript and previous localization of its putative protein product (RN1 immunoreactivity). Those cases where the mRNA and the protein product do not correlate can be explained by the mRNA being expressed in cell bodies, localized distant to the cell fibers (such is the case for the muscle, where the innervating fibers expressing the protein are more abundant than the cell bodies that express the mRNA). Finally, the labeling pattern produced by antibodies generated against the holothurian HgSTARD10 is similar, if not identical, to that of the RN1 antibody, recognizing similar cellular and tissue structures, and nerve fibers. The differences in the protein bands detected in western blot versus those obtained after immunoprecipitation could be simply due to difference in the extraction method used for both experiments. Alternatively, different protein isoforms or posttranslational modifications

(such as phosphorylation) could explain these differences. However, further experiments are required to dissect these possibilities.

HgSTARD10 is the ortholog of mammalian STARD10

START domain-containing proteins are widely conserved through evolution in animals including both deuterostomes and protostomes. In mammals, START proteins are grouped into 6 subfamilies (Alpy et al. 2009). Orthologs from human, flies, nematodes and two echinoderm species grouped in different clades corresponding to at least five subfamilies with no species-specific clustering for most START proteins except for some nematode *C. elegans* orthologs. The phylogenetic analysis also showed that all but one (*S. purpuratus* STARD9) echinoderm subfamily members grouped in the corresponding clade with orthologs of humans and *Drosophila*, thus suggesting that echinoderms have members in at least 4 STARD subfamilies with the STARD9 being a member of a fifth subfamily that was not clearly defined by our analyses.

Our analysis of the HgSTART domain-containing protein recognized by RN1 revealed that it is similar in structure to the mammalian STARD10. It is known that STARD10 belongs to the STARD2/PCTP subfamily of START proteins, which also comprises STARD11, STARD7, and PCTP (Alpy et al. 2009, Clark 2012, Teng et al. 2013). In addition to HgSTARD10, two other START domain-containing proteins were identified in *H. glaberrima* databases: STARD7, also of the STARD2/PCTP subfamily, and STARD13 of the Rho-GAP START group. No echinoderm orthologs were found for STARD14 and STARD15 using the BLAST algorithm suggesting that this subfamily is not evolutionarily conserved in members of the Echinodermata phylum. Advances in genome sequencing and computational tools will further refine the level of conservation of this family of proteins in echinoderms and deuterostomes.

Nervous system specificity

Previous studies from our group showed that RN1 immunoreactivity is localized to the nervous structures of all the holothurian organs assayed (Diaz-Balzac et al. 2007). Similarly, the labeling of the HgSTARD10 antibody described in this report also labels the nervous component of the sea cucumber *H. glaberrima*.

It is interesting that another monoclonal antibody produced against nerve extracts from another echinoderm, the adult starfish *Asterina pectinifera*, also appears to recognize a START domain-containing protein (Murabe et al., 2008). This antibody has been shown to recognize an antigen expressed by a subset of neurons in the nervous system of the echinoderm larva of both sea stars and sea cucumbers labeling cell bodies and fibers (Nakano et al. 2006, Murabe et al. 2008). These cells were similar in shape to serotonergic neurons in the brachiolaria larva arms, and the lateral and oral ganglion (Murabe et al. 2008; Nakano et. al., 2006). It is rather surprising that extracts of adult echinoderm nervous tissue, made independently from two different species, in two different laboratories, both produce antibodies to the same molecule. This strongly suggests that STARD10 is found in large concentrations in the nervous tissue, that the molecule is highly immunogenic or a combination of both.

START-domain expressing proteins are also expressed in the vertebrate nervous system at different developmental stages and in the adult (Chang et al. 2011, Sierra et al. 2003). In fact, members of STARD subfamilies 1 and 6 are differentially expressed in the murine brain (Chang et al. 2010, Chang et al., 2012, Chang et al. 2013). Therefore, it was not surprising to find an abundance of HgSTARD10 mRNA in the radial nerve, being this organ one of the major components of the echinoderm nervous system. The finding confirmed our hypothesis that the STARD10 in *H. glaberrima* is in high abundance in neurons, being the radial nerve the tissue with the highest abundance of protein and mRNA due to the high number of neuronal cell bodies that reside in this organ (Díaz-Balzac, 2007). Moreover, HgSTARD10 is expressed in multiple organs including the longitudinal and circular muscles, hemal system, digestive tract, and mesenteries, probably due to the nervous innervation of the organs.

Novel lipid transfer function in the nervous system

The START domain has been described as a hydrophobic tunnel that forms a pocket for lipophilic ligands such as cholesterol and phosphatidylcholine (Alpy, 2013). The domain is conserved through evolution in plants and animals, serving as the binding interface for lipids that function in a myriad of processes (Soccio and Breslow, 2003; Schrick et al., 2004). A lipid-mediated molecular mechanism of action has been described for some mammalian START domain-containing proteins (Alpy et al., 2013; Christenson et al., 2006; Clark et al., 2012). Some STARD proteins function as lipid sensing proteins while others are common in homeodomain transcription factors in plant species. In addition, proteins from this family are thought to have a role regulating the transcription of genes, supported by the nuclear localization of some members (Schrack et al. 2004, de Brouwer et al. 2002, Olayioye, M. A. et al., 2004). Others are implicated in neurosteroidogenesis (Zwain, 1999) and, possibly, in the development of the nervous system (Chang, 2012).

In view of the conserved sequences of STAR proteins among the different species, it is plausible to hypothesize that sequence similarities also correspond to conserved functions in cellular and molecular processes. In this respect, it may be the case, that the newly characterized HgSTARD10 is also involved in lipid-mediated cellular processes in the various nervous structures associated with diverse organs. Mammalian STARD10 functions as a phospholipid transfer protein, specifically involved in the membrane trafficking of phosphatidylcholine and phosphatidylethanolamine (Olayioye, 2005). This is particularly interesting in view of the possible requirements of neuronal tissue for these two lipids in the growth and maintenance of axons. Thus, it is possible that STARD10 mediates lipid-related biological processes such as intracellular lipid transport, lipid metabolism, and cell signaling events in the nervous system. The expression of this protein during the development of the starfish and the development of the sea cucumber, implicated it as a regulator of lipid-mediated biological processes such as intracellular lipid transport, lipid metabolism, and cell signaling events in the nervous system (Nakano et al., 2006). Based on its wide conservation pattern and conservation during evolution, STARD10 is likely involved in basic cellular functions in neuronal cells. We also hypothesize that phosphorylation could modulate the function of HgSTARD10. Mammalian STARD10 is phosphorylated on its S284 residue in human HEK293T cells (Olayioye, 2007). Mass spectrometry analysis revealed

phosphorylation sites in a peptide that mapped in the C-terminal domain of STARD10. The phosphorylation of these C-terminal residues in the human STARD10 is critical for its functional regulation (Olayioye et al., 2007). In view of these data, a phosphorylation-dependent regulation may be hypothesized for the holothurian ortholog.

In summary, we have determined the antigen recognized by an important nervous system marker for echinoderm tissues as being the holothurian ortholog of STARD10 protein. Members of the STARD protein subfamilies are found among all animal groups and echinoderms appear to have representatives of 5 of the 6 described subfamilies. STARD proteins appear to play multiple, important roles in lipid transport and metabolism. The present work sheds new light into the distribution and evolution of STARD proteins. More importantly, it provides important information for students of echinoderm neurobiology and for those interested in nervous system evolution across species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Immunoprecipitation with the RN1 antibody coupled with tandem mass spectrometry analysis

A) Western blot of the immunopurified proteins showed the two expected bands at 53 and 66 kDa with high intensity signal (IP), but not in the input (IN), beads control (BA) and supernatant after immunoprecipitation (SN). B) Mass spectrometry analysis of the immunoprecipitated proteins identified six different peptides with high level of confidence using a sea cucumber's translated cDNA libraries as the identification databases. All peptides mapped onto a conceptually translated contig from radial nerve and intestine cDNA libraries.

Figure 2. START domain-containing proteins share a high degree of sequence similarity
Multiple sequence alignment START domain-containing protein isoform 10 of the sea cucumber *H. glaberrima*, the starfish *A. pectinifera**, and the sea urchin *S. purpuratus* (ID: XP_790235.3) shows a high degree of conservation of the START domain. The highly conserved region spans 205 residues of the primary structure with 52.4% of pairwise identity among the sequences. *The starfish sequence has only been partially identified (Murabe et al. 2008).

Figure 4. HgSTARD10 expression in *H. glaberrima* organs

A) sqPCR and densitometric analysis of gene transcript expression of HgSTARD10 and NADH (control) in holothurian organs and tissues. B–C) Protein expression assessed by Western blot (B) using the RN1 and tubulin antibodies and densitometric protein quantification identified high abundance of HgSTARD10 in RN and low to mid levels of expression in the other tissues that were analyzed. Protein expression was normalized to the amount of protein assayed. (n>3; RN:radial nerve; Int: intestine; Ant Int:anterior intestine (small descending intestine); Asc Int: ascending intestine (small ascending intestine); Pos Int: posterior intestine (large descending intestine); Eso: esophagus; Mus: muscle (longitudinal); RT: respiratory tree; Hem: hemal system; Mes: mesenteries (intestinal mesentery); Gon: gonads).

Figure 5. Immunohistochemistry and western blot with RN1 and anti-HgSARTD10 sera display similar tissue and molecular staining patterns

A and C) Radial nerve cord was labeled with both RN1 monoclonal antibody (A) or anti HgSTARD10 serum (C) with similar patterns of immunoreactivity. Both antibodies labeled the neuropile of the radial nerve cord (green) with negligible labeling of the neuronal soma. D and F) Nerve plexi in the body wall of the holothurian was labeled by RN1 (B) and anti-HgSTARD10 serum (D). Nuclei in A–D are labeled by DAPI (red). Scale bar: 30 μ m. E) Western blot with the RN1 antibody and sera of mice immunized with recombinant HgSTARD10 identified bands at 53 and 66 kDa in muscle and radial nerve tissue.