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Molecular characterisation of a novel cell surface ADP-ribosyl cyclase from the sea urchin

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Abstract

The sea urchin is an extensively used model system for the study of calcium signalling by the messenger molecules NAADP and cyclic ADP-ribose. Both are synthesized by ADP-ribosyl cyclases but our molecular understanding of these enzymes in the sea urchin is limited. We have recently reported the cloning of an extended family of sea urchin ADP-ribosyl cyclases and shown that one of these enzymes (SpARC1) is active within the endoplasmic reticulum lumen. These studies suggest that production of messengers is compartmentalized. Here we characterize the properties of SpARC2. SpARC2 catalysed both NAADP and cyclic ADP-ribose production. Unusually, the NAD surrogate, NGD was a poor substrate. In contrast to SpARC1, heterologously expressed SpARC2 localized to the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor. Transcripts for SpARC2 were readily detectable in sea urchin eggs and a majority of the endogenous membrane bound activity was found to be GPI-anchored. Our data reveal striking differences in the properties sea urchin ADP-ribosyl cyclases and provide further evidence that messenger production may occur outside of the cytosol.

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Keywords

ADP-ribosyl cyclases; cyclic ADP-ribose; NAADP; sea urchin

1. Introduction

ADP-ribosyl cyclases constitute a family of multifunctional enzymes that can synthesize and metabolise a range of molecules involved in calcium homeostasis [1;2]. The first ADP-ribosyl cyclase to be cloned was a soluble form from *Aplysia* [3]. Two mammalian homologues, CD38 [4] and CD157 [5] have since been identified and more recently a homologue from *Schistosoma* [6]. CD38 has been the most extensively studied family member. This enzyme catalyses cyclic ADPribose production (by cyclization of NAD) and NAADP production (by base-exchange of the nicotinamide moiety in NADP with nicotinic acid) [1]. Both these molecules are potent calcium mobilizing messengers [7]. CD38 can also hydrolyze these molecules [4;8] and catalyze novel reactions that produce adenine homodinucleotides from cyclic ADP-ribose [9]. The latter modulate cytosolic calcium levels through distinct mechanisms. The major activity of CD38 is the hydrolysis of NAD directly to ADP-ribose [4]. This nucleotide stimulates calcium influx via activation of TRPM2 channels [10]. That calcium signals regulate a whole range of cellular processes underscores the physiological importance of these ubiquitous enzymes. Indeed, ADP-ribosyl cyclases have been implicated in cellular processes ranging from temperature stress in sponges [11] to social behaviour in mice [12].

The presence of signal sequences in all ADP-ribosyl cyclase family members renders the catalytic site of the enzyme within the extracellular/luminal face. Indeed, the presence of multiple disulfide bonds limits their location to oxidizing environments. Accordingly, CD38, CD157 and the *Schistosoma* enzyme are predominantly located on the plasma membrane, the latter two via glycosylphosphatidylinositol (GPI) anchors [1;2]. This location poses a topological conundrum for enzymes that produce messenger molecules active in the cytosol. Nevertheless, use of the CD38 knockout mouse has clearly implicated the role of this ADP-ribosyl cyclase in generating cyclic ADP-ribose *in vivo* [13]. This so called “topology paradox” [14] might indicate that messenger synthesis occurs in the extracellular space followed by transport of products into cells [15]. Alternatively, synthesis may occur in a luminal environment following internalization of surface enzymes [16]. Indeed, there are many reports for an intracellular location of CD38 [1;2] although topology constraints still necessitate mechanisms for transport of substrates into, and release from, CD38 expressing organelles.

ADP-ribosyl cyclase activity was first discovered in eggs from the sea urchin [17] and much has been learned using this model organism regarding the mechanism of action of cyclic ADP-ribose and NAADP [18;19]. Although multiple activities have been characterized in cell homogenates [20;21;21], molecular details of the synthetic machinery have been conspicuously lacking. We recently determined the primary structures of three sea urchin ADP-ribosyl cyclases [22]. We provided evidence that SpARC1, the first member of this expanded family, is localized to the endoplasmic reticulum lumen and can gain access to

extra-luminal substrate. These data provide molecular evidence that messenger synthesis may be compartmentalized. In the present study, we report properties of SpARC2. We demonstrate that SpARC2 has unusual substrate preference and is a GPI-anchored plasma membrane protein. Additionally, we find that a substantial fraction of ADP-ribosyl cyclase activity in sea urchin eggs is GPI-anchored. Our data provide further evidence that messenger production may occur in extra-cytosolic compartments.

2. Experimental procedures

2.1. Generation of expression constructs

The full-length coding sequence of SpARC2 was amplified from a *Strongylocentrotus purpuratus* ovary cDNA library, kindly provided by Professor Gary Wessel (Brown University, USA), using the polymerase chain reaction (PCR) and primers SpARC2 1F + 1R (Table 1). The product, which included the ATG codon immediately preceding the predicted start codon, was then cloned into pCS2+ (<http://sitemaker.umich.edu/dlturner.vectors/home>) at the BamHI/XbaI sites, to generate an expression construct encoding for SpARC2 untagged (Fig. 1B). Constructs for *myc* and monomeric RFP (mRFP)-tagged SpARC2 (Fig. 1B) were generated by inserting the appropriate tag after the putative signal peptide between proline 23 and glycine 24. For the single *myc* tagged construct (SpARC2 *myc*), this was achieved by sequence overlap extension [23] using primers SpARC2 1F + 2R to amplify the 5' region of SpARC2 (residues –3 to 69) and primers SpARC2 2F and 1R to amplify the remaining 3' region (residues 70 – 999). The resulting products into which the *myc* sequence had been introduced were assembled in a second PCR using the outer primers SpARC2 1F + 1R, and cloned into pCS2+ at the BamHI/XbaI sites. For the six *myc* tagged expression construct (SpARC2 6 *myc*), the same 5' and 3' regions of SpARC2 were amplified using primers SpARC2 1F + 3R and 3F + 1R, respectively. The products were then sequentially cloned, in frame, at the BamHI/ClaI and ECoRI/XbaI sites of pCS2+ MT (<http://sitemaker.umich.edu/dlturner.vectors/home>). The six *myc* tagged construct, in which the last 37 amino acids were deleted, (SpARC2 C 6 *myc*) was generated similarly, except that primer SpARC2 4R was used in place of primer SpARC2 1R in order to amplify a truncated 3' fragment (residues 70 – 885) containing an introduced STOP codon. For the mRFP-tagged construct (SpARC2 mRFP), PCR products corresponding to the coding sequence of mRFP (generated using mRFP 1F + 1R) and the 3' region of SpARC2 (generated using SpARC2 3F + 1R) were sequentially cloned into pCS2+ MT harboring the 5' SpARC2 sequence (see above). mRFP was amplified from pCS2+ mRFP kindly provided by Dr Masa Tada (UCL) and inserted at the ClaI/ECoRI sites. The 3' SpARC2 product was inserted at the ECoRI/XbaI sites. SpARC1 was amplified using primers SpARC1 1F and 1R and cloned into pCS2+ at the BamHI/XbaI sites to generate SpARC1 untagged. The fidelity of all constructs was verified by DNA sequencing. Specific amplification conditions can be provided upon request. The expression construct harboring GFP-tagged human reduced folate carrier expression was described in [24].

2.2. In Vitro Transcription and Translation

Capped mRNA transcripts were prepared using the mMessage mMachine® kit (Ambion) according to the manufacturer's instructions. SpARC2 harboring plasmids were linearized at

the BstX1 site. mRNA was translated using the rabbit reticulocyte lysate system either in the absence or presence of canine pancreatic microsomes (Promega) as described previously [22].

2.3. Cell Culture

Xenopus laevis eggs were procured and fertilized using standard techniques [25]. Stage VI oocytes were collected from female *Xenopus laevis* and maintained in modified Barth's saline supplemented with 25 µg/ml Gentamicin (Sigma). In some experiments, oocytes were matured *in vitro* with progesterone (1 µg/ml) for 12h at 18°C. Human embryonic kidney (HEK) cells were maintained in DMEM supplemented with 10% (v/v) serum and 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Media was replaced every 3–4 days. Eggs from *Strongylocentrotus purpuratus* were obtained by intracoelemic injection of 0.5 M KCl and collected in to filtered sea water, de-jellied and flash frozen prior to preparation of homogenates.

2.4. Microinjection of *Xenopus laevis* Embryos and Oocytes

Embryos were injected at the 2-cell stage with 0.8 ng of synthetic mRNA per blastomere and incubated in 5% normal amphibian medium [25], containing 3% Ficoll (Sigma), at 14°C. Embryos were collected at the late blastula stage. Oocytes were defolliculated either manually or by incubation with 2 mg/ml Collagenase (Sigma Type 1A) in modified Barth's saline, for 2 hours with agitation. cDNA was microinjected (~8 ng plasmid) directly into the nucleus of the oocyte.

2.5. Transfection of HEK Cells

Cells were seeded either directly into 6-well plates (for western blotting) or onto 25 mm diameter coverslips (for immunocytochemistry). Surfaces were coated with poly-L-lysine (20 µg/ml). Upon reaching ~90% confluency, the cells were transiently transfected with 4 µg of plasmid or DNA (or combination) using Lipofectamine™ 2000 transfection reagent (Invitrogen) and according to the manufacturer's instructions. Subsequent analysis was performed 17h posttransfection.

2.6. Deglycosylation

Embryos were homogenised in a medium (20 µl/embryos) composed of 50 mM NaCl, 10 mM MgAcetate, 1% v/v Triton X-100, 20 mM Tris (pH 7.2) and Complete™ EDTA-free protease inhibitors (Roche). Samples were incubated for 30 min and then clarified by centrifugation at 21,000 × g for 1 min. All the above procedures were performed at 4°C. The homogenates (0.5 embryo equivalents) were then denatured and treated with PNGase F (New England Biolabs) according to the manufacturer's instructions before Western Blot analysis.

2.7. Enzyme Activity Measurements of Heterologously Expressed SpARCs

Xenopus laevis embryos expressing either untagged SpARC1 or SpARC2 were homogenized (4.5 ul/embryo) in a hypotonic medium containing 20 mM HEPES (pH 7.2) and Complete™ EDTA-free protease inhibitors (Roche). The samples were then sonicated

(3 × 5 s bursts) and stored in single use aliquots at −20°C prior to determination of enzyme activity. For baseexchange activity measurements, hypotonic lysates (typically 50 µl) were diluted 5-fold in to a medium composed of 1 mM NADP and 50 mM nicotinic acid (pH ~ 4). For measurement of cyclase activity, the homogenates were diluted 5-fold in medium containing 20 mM HEPES (pH 7.2) and 1 mM NAD or NGD. Reactions were allowed to proceed up to 21 h at room temperature after which the samples were diluted 10-fold with H₂O and particulate material removed by centrifugation (12,000 × g, 1 min). The supernatants were analyzed for nucleotide content by AGMP1 (Biorad) anion exchange HPLC as described [22].

2.8. Secretion Assays and Phosphatidylinositol-specific Phospholipase C Treatment

Media (2 ml) from HEK293 cells expressing SpARC2 6 *myc* or SpARC2 C 6 *myc* was collected 17h post transfection and concentrated (~10–15-fold) using Amicon® Ultra 4 Centrifugal Filter Device (10 kDa molecular weight cut-off). The remaining cells were scraped in to hypotonic medium (see above) and sonicated. In some experiments, the samples were deglycosylated as described for *Xenopus* homogenates. For phosphatidylinositol-specific phospholipase C treatment, HEK cells expressing SpARC2 6 *myc* were washed twice in HEPESbuffered saline (156 mM NaCl, 3 mM KCl, 2mM MgSO₄, 1.25mM KH₂PO₄, 10 mM D-Glucose, 2 mM CaCl₂, 10mM HEPES, pH 7.4) and incubated in the absence or presence of 1 U/ml phosphatidylinositol-specific phospholipase C (Sigma) at 37°C for 1 h. The collected media was concentrated as described above and the cells processed for immunocytochemistry (see below).

2.9. Western Blotting

Homogenates (0.25–0.5 embryo equivalents; 25–50 µg of HEK cell homogenate) or concentrated media from secretion/phosphatidylinositol-specific phospholipase C assays (0.67 – 9.5 cell equivalents) were reduced, separated on NuPAGE® 4–12 % Bis-Tris gels (Invitrogen) and transferred to nitrocellulose filters (ProBlott™, Applied Biosystems) according to standard procedures. The filters were then blocked overnight at 40C with 5 % w/v dried skimmed milk in Tris-buffered saline (25 mM Tris, 137 mM NaCl and 2.7 mM KCl, pH 7.4) containing 0.1 % v/v Tween® 20 (TBS-T) and sequentially incubated with anti-*c-myc* monoclonal antibody (clone 9e10 from Autogen Bioclear; 1/1000 dilution) and anti-mouse IgG conjugated to horse-radish peroxidase (1/1000 dilution) in Tris-buffered saline/Tween supplemented with 2.5 % w/v dried skimmed milk for 1h at room temperature. After each step, the filters were washed with Trisbuffered saline/Tween (3 × 30 min). The resulting blots were developed using the ECL™ Advanced system (Amersham) according to the manufacturer's instructions.

2.10. Immunocytochemistry

Immunolocalization of SpARC2 *myc* and SpARC2 6 *myc* expressed in oocytes was performed as described previously [22]. Briefly, cells were fixed in 1% formaldehyde / methanol at −20°C and sequentially incubated with anti-*c-myc* monoclonal antibody (9E10 Abcam) and anti-mouse IgG conjugated to FITC or Alexa Fluor®568. For immunolocalization of SpARC2 in HEK cells, live cells were labelled with the primary anti-

c-myc antibody. To achieve this, cells were washed twice in HEPES-buffered saline and incubated with the primary antibody (1/100) in HEPES-buffered saline for 1h at 4°C. The temperature was reduced to prevent possible internalization. Unbound antibody was removed by washing coverslips three times in phosphate buffered saline and cells were then fixed in paraformaldehyde (4% w/v) at 25°C for 10 min. Cells were washed again (three times in phosphate buffered saline) and incubated in blocking buffer consisting of phosphate buffered saline supplemented with fetal bovine serum (5% v/v) and bovine serum albumin (1% w/v). Cells were subsequently incubated with secondary antibody (polyclonal, Cy5™-conjugated anti-mouse IgG from goat; Zymed; 1/100 dilution) in blocking buffer at 37°C for 1h, washed three times in phosphate buffered saline and coverslips mounted on slides with 1,4-diazabicyclo[2.2.2]octane (DABCO) and sealed immediately.

2.11. Confocal Microscopy

Images of oocytes and eggs were captured using either a BioRad MRC1024 confocal scanner attached to an Olympus AX70 microscope equipped with a x60 oil-immersion objective or an Olympus IX81 microscope equipped with a x60 oil-immersion objective with an arc-lamp based spinning-disk confocal module (Olympus DSU) with electron-multiplication CCD camera (Hamamatsu, C9100-12). Images of HEK cells were captured using a Zeiss LSM 510 confocal microscope (Welwyn Garden city, Herts, UK) using conventional filter-based fluorescence optics. The respective excitation and emission wavelengths were 488 nm and 505–550 nm for GFP, 543 nm and 560–615 nm for mRFP and 633 nm and >650 nm for Cy5.

2.12. Measurement of endogenous ADP-ribosyl cyclase activity in sea urchin eggs

Egg samples were resuspended at concentration of 25% v/v in a buffer containing 20 mM HEPES and EDTA-free Complete protease inhibitors. The samples were homogenized and baseexchange activity measured following a 10-fold dilution of the homogenate in to assay buffer containing 50 mM nicotinic acid and 1 mM NADP (pH 4). Samples were incubated at room temperature for 1 – 3 h and quenched by 10-fold dilution in to H₂O followed by heating to 60°C for 5 min. Samples were centrifuged for 3 min at 21000 × g to remove particulate material and the supernatants analyzed for NAADP content by HPLC as described [22]. To separate membrane bound and soluble activity, the homogenates were diluted two-fold, and centrifuged at 100,000 × g for 1h at 4°C. The membranes were resuspended in the original volume with homogenisation buffer and equivalent volumes of membranes and supernatant fractions assayed. In some experiments, the diluted homogenates were treated with or without 1U/ml phosphatidyl inositol-specific phospholipase C for 2 h at room temperature prior to fractionation.

2.13. Reverse-Transcription PCR (RT-PCR)

Total RNA was isolated from *Strongylocentrotus purpuratus* eggs using the RNeasy kit (Qiagen) according to the manufacturer's instructions. First strand synthesis was performed using the ImProm-II™ kit (Promega) according to the manufacturer's instructions using oligo-dT primers. Typically, 1 µg of RNA was reverse-transcribed in a 20 µl reaction. In control experiments, the reverse-transcriptase was omitted from the reaction. PCRs

consisted of an initial 2 min denaturation step at 94°C followed by 35 cycles of denaturation (30s, 94°C), annealing (30 s, 50°C) and extension (1 min, 72°C) using Platinum® *Taq* DNA Polymerase (Invitrogen). The sequences of the primers used for detection of SpARC2 (5F + 5R) are listed in Table 1.

3. Results

3.1. Predicted structure of SpARC2 and heterologous expression

We recently determined the coding sequence of SpARC2, a member of a novel family of sea urchin ADP-ribosyl cyclases [22]. As schematically depicted in Fig. 1A, SpARC2 is predicted an N terminal signal peptide, 5 N-linked glycosylation sites and a C terminal glycosylphosphatidylinositol [22]. In order to functionally characterize SpARC2, the indicated series of expression constructs were generated (Fig. 1B). In the first set of experiments, we expressed SpARC2 6 *myc* in *Xenopus* embryos - a heterologous expression system we have used previously to define the properties of SpARC1 [22]. Western blot analysis with an anti-*myc* antibody revealed a major band corresponding to a molecular weight of 63 ± 4 kDa ($n = 3$) (Fig. 1B). We also noted diffuse higher molecular bands ($63 - 73$ kDa) and in some preparations a less abundant lower molecular band (44 kDa). Given that the predicted molecular weight of SpARC2 6 *myc* is 47 kDa, we tested whether the higher molecular weight bands corresponded to glycosylated forms of the protein. Migration of SpARC2 6 *myc* was therefore compared following treatment with PNGase F. As shown in the last two lanes of Fig. 1C, there was a clear reduction in apparent molecular weight of the expressed protein following deglycosylation to 54 kDa ($n = 2$). Glycosylation of (untagged) SpARC2 was also confirmed *in vitro* using the rabbit reticulocyte translation system. As shown in Fig. 1D, a product of 33 KDa was obtained for SpARC2 untagged in close agreement with its theoretical molecular weight of 36 kDa, whereas in the presence of microsomes, the mobility was substantially decreased. Taken together these data indicate SpARC2 is N-glycosylated.

3.2. SpARC2 catalyses production of NAADP and cyclic ADP-ribose but not cyclic GDPribose

We next characterized the catalytic properties of SpARC2. To achieve this, we initially incubated homogenates prepared from embryos expressing SpARC2 untagged with saturating concentrations of NADP and nicotinic acid under acidic conditions. These conditions favour production of NAADP by the base-exchange reaction. Indeed, as shown in Fig 2A, NAADP was readily produced in a time dependent manner. To determine whether SpARC2 could catalyse the cyclisation reaction, the homogenates were incubated with NGD, a commonly used NAD, surrogate, under near neutral conditions. Somewhat surprisingly, cyclic GDP-ribose production was either not detectable or at the limit of detection (Fig. 2A, open circles). Analysis of the initial rates of production from several similar experiments are summarised in Figure 2C. The lack of activity was not due to the inability to detect the product because untagged SpARC1 expressed in parallel readily cyclized NGD (Fig. 2B–C) confirming our previous analysis using *myc*-tagged SpARC1 [22]. Nor was the lack of production due to an inherent inability of SpARC2 to catalyze the cyclisation reaction since cyclic ADP-ribose production using the natural substrate NAD

was measurable although production was modest relative to that of NAADP (Fig. 2C). Similar results using NAD were obtained for SpARC1 (Fig. 2C). These data indicate that in marked contrast to SpARC1, NGD is a poor substrate for SpARC2. The clear difference in catalytic properties between isoforms is further highlighted in Fig. 2D where we compared the ratio of initial rates of cyclic GDP-ribose and NAADP production. Note that this measure of relative activity is independent of absolute expression levels between embryo batches. This analysis reveals that SpARC2 is substantially more efficient at producing NAADP over cyclic GDP-ribose than SpARC1. In contrast, cyclic ADP-ribose production relative to NAADP production is comparable for the two isoforms.

3.3. SpARC2 localizes to the plasma membrane

To define the subcellular distribution of SpARC2, we expressed the *myc*- and mRFP-tagged constructs in *Xenopus* oocytes. These huge cells offer exquisite resolution of subcellular structures. Immunocytochemical analysis of both SpARC2 *myc* (Fig. 3A) and SpARC2 6 *myc*, (Fig. 3B) revealed clear cell surface labelling as evidenced by colocalization of both constructs with a plasma membrane marker. SpARC2 distribution was also determined in progesterone-matured oocytes. In these cells, it was evident that SpARC2 6 *myc* was internalised (Fig. 3C) consistent with the marked endocytosis of the plasma membrane during maturation [26]. Use of the mRFP tagged construct allowed us to monitor this internalisation process in real time. As shown in Fig. 3D, there was a progressive loss in plasma membrane fluorescence of both SpARC2 mRFP and the plasma membrane marker (see also Supplementary Movie). These data indicate that in marked contrast to SpARC1, SpARC2 is an ectoenzyme.

3.4. SpARC2 is a glycosylphosphatidylinositol-anchored protein

To determine whether association of SpARC2 with the plasma membrane was mediated via a GPI anchor, we took two approaches. In the first, we used a 6 *myc* tagged truncated construct lacking the last 37 C terminal amino acids encompassing the predicted anchoring site (SpARC2 C 6 *myc*; Fig. 1B). We then compared secretion from HEK cells transfected with either the fulllength construct (SpARC2 6 *myc*) or the truncated one. As shown in Fig. 4A, secretion of SpARC2 6 *myc* was not readily detected (although a weak signal was consistently noted). In marked contrast, SpARC2 C 6 *myc* secretion was clearly detectable as a diffuse band of ~ 68 kDa (Fig. 4A). Blotting of the cell fractions in parallel revealed comparable expression levels of the two constructs (Fig. 4A). The molecular weights of the major bands were 64 and 59 kDa for SpARC2 6 *myc* and SpARC2 C 6 *myc*, respectively. Interestingly, the molecular mass of the secreted truncated protein (~ 68 kDa) was higher than the major protein detected within the cell fraction (59 kDa). In order to further characterize the secreted products, we treated the media samples with PNGase F (Fig. 4A). Deglycosylation reduced the molecular weight of the secreted product from the SpARC2 C 6 *myc*-expressing cells to 49 kDa and therefore close to the predicted (un-glycosylated) molecular weight of this construct (43 kDa). This treatment also revealed a faint, but well resolved 53 kDa secreted protein in cells transfected with the full length SpARC2 construct (Fig. 4A). Again, this mass was close to that expected (47 kDa) indicating that secretion of the full length construct did occur, but that this was modest relative to the truncated construct. These data suggest that membrane association of

SpARC2 is mediated by its C terminus and that secreted products undergo more extensive glycosylation than the cellular pool.

In the second approach, we treated live HEK cells expressing SpARC2 6 *myc* with bacterial phosphatidylinositol-specific phospholipase C, which cleaves GPI anchors. As shown in Fig. 4B, Western blot analysis of media collected from cells that had been treated with the enzyme revealed the presence of SpARC2 as a diffuse band of ~ 66 kDa. In converse experiments, we examined the effect of phospholipase C on cell surface expression of SpARC2 6 *myc*. Results from immunocytochemical analysis (Fig. 4C–D) demonstrate a reduction in SpARC2 6 *myc* immunoreactivity after phospholipase C treatment. As expected, enzyme treatment did not affect expression levels of the co-expressed plasma membrane marker (reduced folate carrier) consistent with its anchoring via multiple transmembrane domains (Fig. 4C–D). Collectively, the data in Fig. 4 indicate that SpARC2 is a GPI-anchored ectoenzyme.

3.5. Endogenous ADP-ribosyl cyclase activity in sea urchin eggs

In the final set of experiments, we characterized endogenous ADP-ribosyl cyclase activity in sea urchin eggs, a widely used preparation for the study of NAADP and cADPR signalling [18]. Crude egg homogenates incubated with NADP and nicotinic acid readily produced NAADP at an initial rate of 4.6 ± 0.36 nmol/h/mg ($n = 7$). Measurement of activity in homogenates that had been fractionated by ultracentrifugation revealed that a majority of synthesis occurred in the membrane fraction (Fig. 5A, left). To determine whether any of the membrane-associated activity was GPI-anchored, we performed experiments using homogenates that had been pretreated with bacterial phospholipase C prior to fractionation. As shown in Fig. 5A (right), following treatment, there was a more than 2-fold reduction in activity in the membrane fraction with a corresponding increase in the soluble fraction. Pooled data from several similar experiments is shown in Fig. 5B. These data reveal robust GPI-anchored ADP-ribosyl cyclase activity in these cells. Results from RT PCR experiments (Fig. 5C) confirmed the presence of SpARC2 transcripts in eggs as evidenced by amplification of a band of the expected size (528 bp). No signal was obtained using a mock template prepared in the absence of the reverse transcriptase (Fig. 5C).

4. Discussion

Despite the importance of the sea urchin as a model system for the study of inositol trisphosphate-independent calcium mobilization, molecular details of the target calcium channels and synthetic machinery are seriously lacking. Here we report, for the first time, the properties of SpARC2, a member of a recently described expanded family of sea urchin ADP-ribosyl cyclases. We find that SpARC2 has an unusual substrate preference and is GPI-anchored plasma membrane protein. Additionally, we show that GPI-anchored ADP-ribosyl cyclase activity accounts for a substantial fraction of endogenous activity in sea urchin eggs where SpARC2 is likely expressed.

NGD is widely used as a NAD surrogate to measure cyclase activity because its conversion is more efficient than that of the natural substrate NAD (at least for CD38), and the fact that the formed product is more resistant to hydrolysis than cyclic ADP-ribose [27]. The

preference of NGD over NAD for the cyclisation reaction has attracted much attention since delineating the underlying mechanism is likely to provide major insight in to the multifunctional nature of ADP-ribose cyclases [28]. One striking feature of SpARC2 is its poor ability to cyclize NGD (Fig. 2). ADP-ribose cyclases that do not utilise NGD have previously been characterized in sub cellular fractions from Jurkat T lymphocytes [29], human peripheral blood mononuclear cells [30] and brain from CD38 deficient mice [31]. In all cases however the molecular identity of the enzyme is not known. Consequently this is the first report identifying an ADP-ribose cyclase at the molecular level for which NGD is a poor substrate. Our data reinforce the cautionary note of Lund and colleagues who have stressed that GDP- and ADP-ribose cyclase activities are not always equivalent [32] as further highlighted by the *Schistosoma* enzyme which favours NGD cyclization over that of NAD [6]. Future studies comparing the structures of SpARC2 with cyclases that can catalyze cyclic GDP ribose production, in particular SpARC1, should prove informative with respect to the unusual divergence in substrate preference identified here.

Our localisation studies show that SpARC2 is a plasma membrane protein (Fig. 3). Thus, in stark contrast to SpARC1, which is an intracellular ADP-ribose cyclase, SpARC2 is an ectoenzyme very much like CD38, CD157 and the *Schistosoma* protein. In the *Xenopus* system, the protein was demonstrably internalised during bulk endocytosis that occurs during oocyte maturation [26]. These data are important as they provide direct evidence that SpARC2 is localized to the plasma membrane. Indeed, we find that membrane association of SpARC2 is mediated by its C terminus, which contains a consensus GPI anchor sequence, and that the protein can be released by bacterial phospholipase C treatment. Thus, in common with CD157 (and the *Schistosoma* enzyme), SpARC2 is GPI-anchored suggesting that it may represent the orthologue of mammalian CD157. Indeed, SpARC2 contains an N terminal signal peptide (like CD157) as opposed to a signal anchor (as found in CD38) although overall sequence similarity between SpARC2 and CD157/CD38 is similar at 30–40 % (see [22]). Interestingly, we found that both secreted SpARC2 and that released by bacterial phospholipase C treatment were of higher apparent molecular weight than that of the cellular pool. The latter may then represent an incompletely glycosylated SpARC2 possibly resulting from overexpression.

Previous studies have identified ADP-ribose cyclase activities in both sea urchin eggs and sperm [20, 21;21]. In egg homogenates, base-exchange activity is predominately found in membrane fractions [20;21]. Our data confirm this and importantly show that a majority of the activity can be released by phospholipase C treatment (Fig. 5). These data indicate the presence of a GPI-anchored ADP-ribose cyclase, likely SpARC2, the transcripts for which are readily detectable in eggs (Fig. 5C). At this stage however we can not rule other GPI-anchored ADP-ribose cyclases contributing to this activity. Residual activity after phospholipase C treatment also suggests that presence of an additional membrane bound ADP-ribose cyclase(s) in the egg possibly a cyclic AMP-sensitive isoform described previously [21]. Membrane bound baseexchange activity has also been described in sea urchin sperm homogenates [33]. Intriguingly, Vasudevan et al have recently provided evidence using intact sperm that this activity is localized to the cell surface [34]. In that study, cyclization of both NAD and NGD was not detectable. Thus, NAADP production by

sperm is unlikely to be ascribable to SpARC2 since despite its common location, SpARC2 is capable of cyclizing NAD (Fig. 2).

In the sea urchin egg there is clear evidence for a soluble (presumed cytosolic) ADP-ribosyl cyclase that is cyclic GMP sensitive [20, 21;35] and which likely contributes to the calcium wave at fertilisation [36]. The molecular identity of this enzyme however remains mysterious. The present study combined with our previous data demonstrating SpARC1 activity within the endoplasmic reticulum lumen [22] provides evidence that messenger production may also occur outside of the cytosol. Whether SpARC2 at the cell surface can gain access to its substrates however remains to be established but for SpARC1 this appears to be the case despite its compartmentalisation [22]. One possibility, is that SpARC2 may produce messengers upon internalization perhaps during fertilisation or at later stages of development. Indeed, experimental support for internalisation of mammalian CD38 in to endosomes has been obtained [16]. It is tempting to speculate that if SpARC2 is internalized in to an acidic environment of the endosomal system (which would favour the base-exchange reaction) then it may preferentially produce NAADP. Conversely, SpARC1 situated in a luminal environment at neutral pH (which would favour the cyclisation reaction) might predominantly synthesize cyclic ADPR-ribose. Calcium mobilizing messenger production may therefore be controlled by distinct isoforms targeted to different subcellular locales.

In conclusion, we provide new molecular details of the ADP-ribosyl cyclase complement of the sea urchin lending further support to the notion that messenger synthesis may be compartmentalized.

Supplementary Material

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Figure 1. Predicted structure of SpARC2 and heterologous expression

A, Schematic representation of SpARC2. Positions of the putative signal peptide, glycosylation sites (Y) and glycosylphosphatidylinositol anchor is indicated. B, Expression constructs used throughout this study. The introduced tags (light shading) are indicated. C, Western blot of homogenates prepared from *Xenopus* embryos expressing SpARC2 6 *myc*. The right hand lanes show the effect of mock (–) and PNGase F (+) treatment on migration of SpARC2 6 *myc*. Blots were probed with an anti *myc* antibody. D, *In vitro* translation of SpARC2 untagged in the absence (–) and presence (+) of microsomes.

Figure 2. SpARC2 catalyzes production of NAADP and cyclic ADP-ribose but not cyclic GDP-ribose

A – B Time course showing production of NAADP and cyclic GDP-ribose by homogenates prepared from *Xenopus* embryos injected with untagged SpARC2 (A) or SpARC1 (B). C, Pooled data (from 3–4 independent embryo preparations) quantifying the initial rate of production of NAADP, cyclic GDP-ribose and cyclic ADP-ribose by the indicated expressed protein. D, Comparison of the ratio of cyclisation to base-exchange activities for SpARC2 and SpARC1 using NGD or NAD as substrates.

Figure 3. SpARC2 localizes to the plasma membrane

Immunocytochemical localization of recombinant SpARC2 *myc* (A) and SpARC2 6 *myc* (B) in *Xenopus* oocytes coexpressing GFPtagged human reduced folate carrier (HsRFC, green). The plane of the confocal images is indicated. C, Localisation of SpARC2 6 *myc* in progesterone matured oocytes. D, Fluorescence images of live oocytes expressing SpARC2 mRFP (red) and GFP-tagged human reduced folate carrier (green) at the indicated times (in h) after progesterone addition. A plot of the average fluorescence of the two proteins during maturation is shown on the right. M, See supplementary Movie 1 for full time-course.

Figure 4. SpARC2 is a glycosylphosphatidylinositol-anchored protein

A, HEK cells were transfected with expression constructs encoding for 6 myc tagged full-length SpARC2 (SpARC2 6 myc) or a truncated construct lacking 37 amino acids at the C terminus (SpARC2 ΔC 6 myc). Western blot analysis of equivalent cell fractions and media is shown. In the last two lanes, the media samples were treated with PNGase F prior to analysis. B, HEK cells were transfected with SpARC2 6 myc and SpARC2 expression levels determined in the media (3.3 cell equivalents) by Western blotting following a 1 h incubation with no additions or addition of bacterial phospholipase C (+). C, Confocal

images showing expression of cell surface SpARC2 6 *myc* (red) and GFP-tagged human reduced folate carrier (HsRFC, green) following mock treatment or treatment with bacterial phospholipase C for 1 h (+ PI-PLC). The average fluorescence intensities of 60 cells (from 6 independent fields of view) are shown in D.

Figure 5. Endogenous ADP-ribosyl cyclase activity in sea urchin eggs

A, Time course for production of NAADP by membrane or soluble fractions prepared from mock treated homogenates (left; - PI-PLC) or homogenates treated with bacterial phospholipase C (right; + PIPLC). B, Pooled data from 4 independent experiments quantifying the initial rate of NAADP production in the indicated preparations. C, Agarose gel electrophoresis analysis of PCR products amplified using egg cDNA prepared in the

absence (–) or presence (+) of reverse transcriptase (RT). Migration of molecular size markers (in bp) is shown to the left.

Table 1

Primer sequences

Introduced restriction sites are underlined.

Primer	Sequence
SpARC2 1F	5'-CACCAGATCTATGATGAATCTTCTCCGACG-3'
SpARC2 1R	5'-GCTCTAGAGTCAATCAATTTTGCAACCAATTTAGCA-3'
SpARC2 2F	5'-GAGCAAAAGCTCATTTCTGAAAGAGGACTTTGGGACCAGGAACCCACATGG-3'
SpARC2 2R	5'-CAAAGTCTCTTCAGAAATGAGCTTTTGCTCTGGCAAGGTATAGGCAACAGT-3'
SpARC2 3F	5'-CACCCGAATTCAGGACCAGGAACCAATGG
SpARC2 3R	5'-ACCCAATCGATGTGGCAAGGTATAGGCAACAGT-3'
SpARC2 4R	5'-GCTCTAGAGTCAATTCCTGCTGCTGTATGG-3'
mRFP 1F	5'-CACCATCGAATTTATGGCCTCTCCGAGG-3'
mRFP 1R	5'-CCTTGAAATTCAGGGCGCCGGTGGAGT-3'
SpARC1 1F	5'-CACCAGATCTATGGGCATCTACACCATATTCA-3'
SpARC1 1R	5'-GCTCTAGAGTTATAGGGTAGTAGATATTGTATTCCAAACC-3'
SpARC2 5F	5'-GCGATGTCAACTGTGATGCT-3'
SpARC2 5R	5'-CTCACAAAGTGTGCTGAGACC-3'