

## RESEARCH HIGHLIGHT

# Multitasking rSp0032 has anti-pathogen binding activities predicting flexible and effective immune responses in sea urchins mediated by the Sp185/333 system

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The purple sea urchin, *Strongylocentrotus purpuratus*, possesses a sophisticated innate immune system that responds to microbes effectively by swift expression of the highly diverse *Sp185/333* gene family. The encoded *Sp185/333* proteins show significant sequence diversity and are predicted to have anti-pathogen functions. To address the anti-pathogen hypothesis, functional analysis of a recombinant *Sp185/333* protein, rSp0032, shows that it exhibits specific binding to a marine *Vibrio* species and to Baker's yeast but not to two *Bacillus* species. rSp0032 binds to lipopolysaccharide (LPS),  $\beta$ -1,3-glucan and flagellin but not to peptidoglycan. rSp0032 binding to LPS is competed by LPS,  $\beta$ -1,3-glucan and flagellin but not by peptidoglycan. *In silico* predictions suggest that rSp0032 is intrinsically disordered and its multiple binding targets suggest adoption of different conformations for binding to different PAMPs and pathogens. Based on rSp0032 binding to a range of targets, and that hundreds of different isoforms of *Sp185/333* proteins are expressed in individual sea urchins, each may have different binding activities imparting a wide range of overlapping binding targets by this family of immune response proteins. The outcome may be very effective host protection against a broad array of potential pathogens in the marine environment.

**Keywords:** bacterial binding; PAMP binding; immune response protein; intrinsically disordered protein; *Strongylocentrotus purpuratus*

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## Introduction

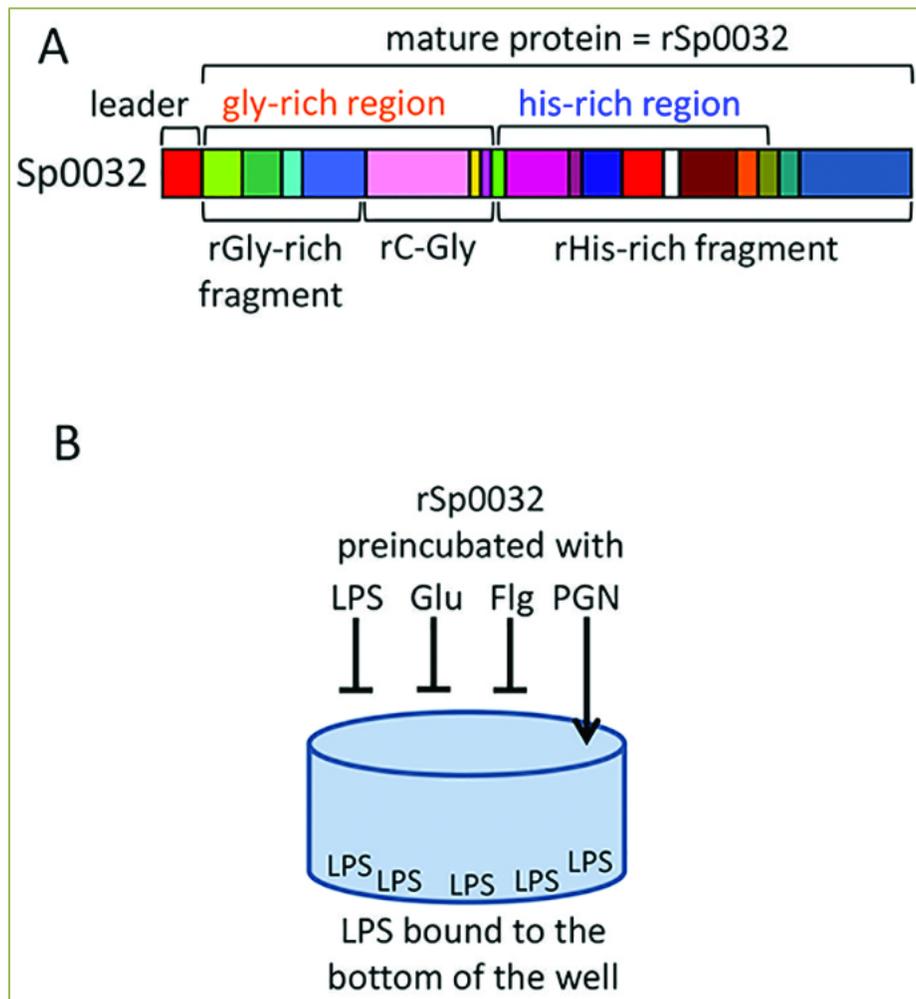
Echinoderms lack adaptive immune capabilities (reviewed in [1, 2]) but have sophisticated innate immune functions with several expanded gene families encoding antigen recognition and effector molecules that likely function in effective pathogen protection [3-5]. One of the immune response gene

families in the purple sea urchin is a set of unique genes called *Sp185/333* [3, 4, 6-9] that are significantly upregulated in response to immune challenges from bacteria and pathogen associated molecular patterns (PAMPs) [10-13]. The genes and deduced proteins have intriguing sequence diversity, which is consistent with putative immune defense functions. The mature proteins have tandem and interspersed repeats, and

**Table 1. Matches to proteins by MS/MS in Sp185/333<sup>+</sup> bands after rSp0032 incubation with target cells**

Sp185/333 <sup>+</sup> band size <sup>1</sup>	rSp0032 alone	rSp0032 & <i>Bacillus</i> <sup>2</sup>	rSp0032 & <i>Vibrio</i>	rSp0032 & Yeast
37 kDa	Sp185/333	-- <sup>3</sup>	--	--
50 kDa	--	--	--	Sp185/333
60 kDa	--	--	Sp185/333 & flagellin	--
80 kDa	--	--	Sp185/333	--

<sup>1</sup>Sp185/333<sup>+</sup> bands were identified from a Western blot evaluated with anti-Sp185/333 antibodies that was run in parallel with the gel that was evaluated by tandem MS/MS. <sup>2</sup>*Bacillus subtilis* or *B. cereus*. <sup>3</sup>No bands of this size were observed.



**Figure 1. A. Predicted Sp0032 protein structure.** The mature Sp0032 protein is composed of a mosaic of elements (colored blocks) that are defined by gaps in alignments<sup>[13]</sup>. The deduced Sp0032 sequence predicts a leader (indicated), which is likely cleaved from the mature protein plus a gly-rich region (orange text) and a his-rich region (blue text). The recombinant fragments of rSp0032 expressed in *E. coli* are labeled as the rGly-rich fragment, rC-Gly, and the rHis-rich fragment. rSp0032 and the rHis-rich fragment are expressed in *E. coli* without the leader. The figure is modified from<sup>[13, 14]</sup>. **B.** rSp0032 binds specifically to LPS,  $\beta$ -1,3-glucan and flagellin but not to PGN. When rSp0032 is pre-incubated with LPS (from *E. coli*),  $\beta$ -1,3-glucan (Glu, from yeast) or flagellin (Flg, from *Salmonella typhimurium*), this blocks binding to LPS that is bound to the well. However, when rSp0032 is pre-incubated with PGN (from *Bacillus subtilis*), rSp0032 binding to LPS in the well is not blocked.

optimal alignments that define 25 to 27 recognizable blocks of sequence (depending on the alignment<sup>[12, 14]</sup>) known as *elements* (Fig. 1A). Elements are present in mosaic combinations that result in 51 recognizable *element patterns* in both the second of two exons in the gene and in the

deduced, mature proteins (reviewed in<sup>[7, 8]</sup>). Although element patterns impart a significant level of sequence diversity, the Sp185/333 proteins show a common structure composed of an N-terminal hydrophobic leader, a glycine (gly)-rich region, a histidine (his)-rich region, and a

C-terminal region (Fig. 1A)<sup>[13]</sup>. The sequence diversity of the proteins plus the swift gene expression in response to immune challenge<sup>[10, 12, 13]</sup> and extensive variation in protein arrays following pathogen exposure<sup>[15, 16]</sup> have led to the hypothesis that Sp185/333 proteins have immunological functions (reviewed in<sup>[7, 8]</sup>). Accordingly, the functional analysis of a recombinant Sp185/333 protein called rSp0032 (Fig. 1A) was recently reported by Lun *et al.*<sup>[17]</sup> and shows multitasking binding activities towards bacteria, yeast, and PAMPs.

## Results

### rSp0032 Binds to *Vibrio diazotrophicus* and Yeast *in vitro*

A subset of native Sp185/333 (natSp185/333) proteins can be isolated from the coelomic fluid (or body fluid) of adult sea urchins by nickel affinity chromatography due to the multiple histidines in the his-rich region<sup>[16]</sup>. The protein diversity and tendency for multimerization of the natSp185/333 proteins<sup>[15, 16, 18]</sup> makes the functional evaluation difficult for these proteins. Consequently, expression of a recombinant Sp185/333 protein that could be evaluated in the absence of other variants was essential to begin to understand the activities of this protein family. Although several cDNAs encoding a range of different Sp185/333 isoforms were tested for expression in *E. coli*, only a single recombinant called rSp0032 (Fig. 1A) did not kill the cells and could be isolated. rSp0032 was first evaluated for anti-pathogen activity by incubation with foreign cells and bound to the marine bacteria, *Vibrio diazotrophicus*, and to Baker's yeast, *Saccharomyces cerevisiae*, but did not bind to either *Bacillus subtilis* or *B. cereus*. Western blots showed that rSp0032<sup>+</sup> bands were larger than expected after binding to *Vibrio* or yeast, suggesting that rSp0032 either i) dimerized or multimerized, which has been observed for natSp185/333 proteins<sup>[15, 16, 18]</sup>, ii) that it bound to *V. diazotrophicus* and yeast proteins that were not dissociated by SDS-PAGE preparation, or iii) a combination of both. To differentiate among these possibilities, rSp0032<sup>+</sup> bands were evaluated by tandem mass spectroscopy (MS/MS) and matches to rSp0032 were identified from bands that corresponded to 37 kDa, which is the expected size of rSp0032 (Table 1). Furthermore, results from the 80 kDa band after incubation with *V. diazotrophicus* also matched to rSp0032, suggesting dimerization rather than binding to target molecules. The 50 kDa band resulting from incubation with yeast only yielded matches to rSp0032. Because 50 kDa is neither the expected monomer nor dimer size of rSp0032, the recombinant protein may have bound to a yeast component that could not be detected by MS/MS. The 60 kDa band after incubation with *V. diazotrophicus* matched to both rSp0032 and to *Vibrio* flagellin suggesting

that rSp0032 may associate tightly with flagellin, a known immune activating PAMP.

### rSp0032 Fragments Show Altered Binding to Bacteria and Yeast

The standard Sp185/333 protein structure has three regions with an N-terminal gly-rich region, a central region at the C-terminal end of the gly-rich region (C-Gly), and a C-terminal his-rich region (Fig. 1A). We have speculated that these regions in the natSp185/333 proteins may have different functions, partly based on differences in amino acid content, and partly based on evidence that many mRNAs appear to be edited and encode truncated proteins without the his-rich region<sup>[12, 16, 19]</sup>. To address this question, recombinant rGly-rich and rHis-rich fragments were expressed in *E. coli* and appeared on Western blots as monomers of the expected size. However, rather than the expected 5 kDa size for the rC-Gly fragment, it appeared as large multimers of 25 kDa and 60 kDa that could not be dissociated to monomers. This suggested that the C-Gly region may mediate dimerization and multimerization that has been repeatedly observed for the Sp185/333 proteins<sup>[15, 16, 18]</sup>. When the rGly-rich and rHis-rich fragments were mixed with bacteria or yeast, both bound to *V. diazotrophicus* in addition to both *Bacillus* species and to yeast, but did not show multimerization as observed for the full length rSp0032. These results demonstrated that both the rGly-rich and rHis-rich fragments could bind to foreign cells and showed expanded binding to *Bacillus* to which rSp0032 did not bind indicating that the three regions of rSp0032 appear to have different functions and show altered activities when separated. This suggested that the three regions of rSp0032 likely function together to initiate more restricted or specific targeting of foreign cells plus multimerization events.

### rSp0032 Shows Saturable Binding to *Vibrio diazotrophicus* and Yeast

rSp0032 bound tightly to *V. diazotrophicus* and yeast, however it was not known whether this was a specific and saturable interaction of the proteins with discrete binding sites. When *V. diazotrophicus*, the *Bacillus* species, or yeast were mixed with increasing concentrations of rSp0032, saturable binding was observed for *V. diazotrophicus* that plateaued at 0.15  $\mu\text{M}$  ( $K_d = 0.2 \text{ nM}$ ), but no measurable binding was detected for the *Bacillus* species. Alternatively, non-linear binding was observed for yeast with two curves that correlated with two concentrations of rSp0032. The first curve ( $\leq 0.4 \mu\text{M}$  rSp0032) suggested strong binding ( $K_d = 1.7 \text{ nM}$ ) and the second curve ( $\geq 0.4 \mu\text{M}$ ) indicated moderately strong binding ( $K_d = 0.8 \mu\text{M}$ ) with a saturable binding plateau at  $\sim 1 \mu\text{M}$ . Not only did these results suggest

**Table 2. Binding competition for yeast among the recombinant fragments and rSp0032 indicates an expansion of binding sites by the rGly-rich fragment**

Binding evaluated for	Competitor	Binding decreased by
rGly-rich fragment	rHis-rich fragment	40%
rHis-rich fragment	rGly-rich fragment	40%
rGly-rich fragment	rSp0032	40%
rHis-rich fragment	rSp0032	100%

specific binding to finite numbers of sites, but that the binding sites on *V. diazotrophicus* and yeast were likely quite different as to type of molecule and their distribution on the target cell surfaces.

rSp0032 Binding to *Vibrio diazotrophicus* and Yeast is tight and specific

One of the expected attributes of an effective immune response protein is that it should not dissociate from its target once bound. To verify this concept, when *V. diazotrophicus* and yeast were fully saturated with rSp0032 labeled with fluorescein (rSp0032-FITC), they did not decrease their fluorescence level upon incubation with increasing concentrations of unlabeled rSp0032. This indicated that once bound, rSp0032 could not be displaced from the target cell in agreement with the strong saturable binding results described above.

Measurements of low levels of rSp0032 dissociation from target cells were followed by an analysis of whether binding to the finite number of sites was specific. Binding competition with mixtures of rSp0032-FITC mixed with increasing concentrations of unlabeled rSp0032 showed that the level of rSp0032-FITC bound to *V. diazotrophicus* decreased as the competitor concentration increased. Similar results were obtained from binding competition on yeast, although results showed two competition curves that correlated exactly with results for saturation binding (above and below 0.4  $\mu$ M rSp0032). This suggested either two phases of binding or two types of binding sites on yeast with different binding coefficients. These results demonstrated that not only did rSp0032 bind to *V. diazotrophicus* and yeast with saturable kinetics, but that the binding sites on the cell surfaces were specific.

rGly-rich and rHis-rich Fragments have Different Characteristics for Binding to Yeast

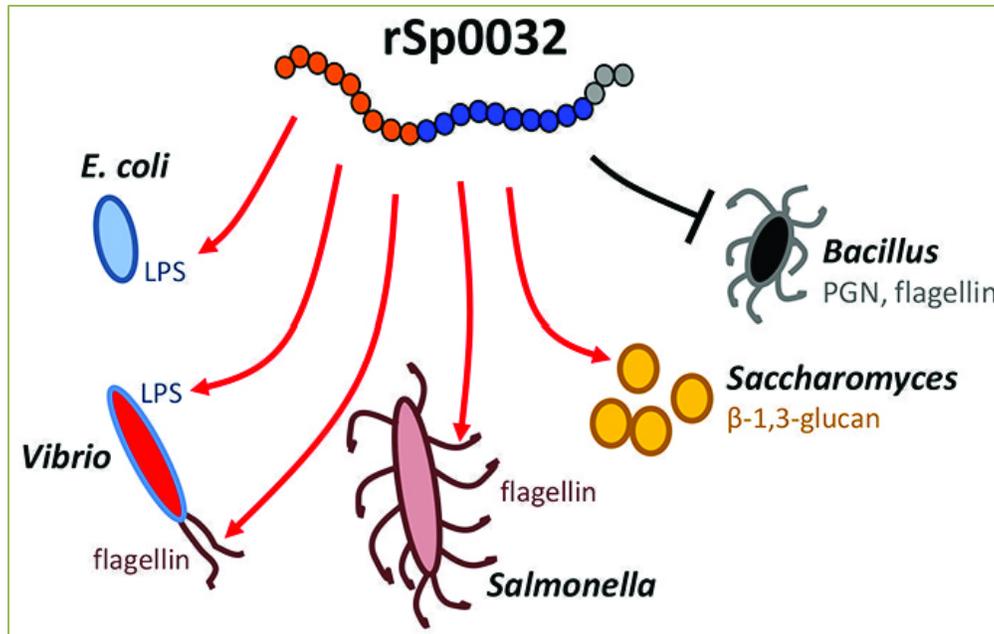
The gly-rich region and the his-rich region of rSp0032 are quite different with regard to the complement of amino acids. The gly-rich region is composed of 30% glycines with no histidines, whereas the his-rich region has 15% glycines and ~15% histidines, which has been the basis for the speculations on different functions for these two regions in Sp185/333 proteins<sup>[17]</sup>. Consequently, the binding

characteristics of the rGly-rich and rHis-rich fragments were tested for binding to yeast. As the concentration of the rGly-rich fragment increased, the amount of bound protein on yeast plateaued at 1.1  $\mu$ M, whereas binding for the rHis-rich fragment plateaued at 0.59  $\mu$ M. The moderately strong binding affinities of the rGly-rich fragment ( $K_d = 1.5 \mu$ M) and the rHis-rich fragment ( $K_d = 5.1 \mu$ M) to yeast were within the same order of magnitude but showed different saturation characteristics and had lower affinity compared to the full length rSp0032 ( $K_d = 0.8$  nM). The different binding characteristics towards yeast suggested either different activities for the two regions of the full length protein that, when separated, either functioned less effectively in binding to yeast, or that the rGly-rich fragment may have bound to more sites than the rHis-rich fragment.

When the two fragments were used in competition with each other for binding to yeast, each decreased binding by the other by 40% (Table 2) suggesting that they bound to distinct but overlapping sites. When the full-length rSp0032 was employed as the competitor, binding to yeast by the rGly-rich fragment was decreased by 40%, whereas binding by the rHis-rich fragment was reduced by 100% (Table 2). These results suggested that the full-length and the rHis-rich fragment bound to the same sites but that the rGly-rich fragment had additional sites on yeast that were not bound by either the rHis-rich fragment or by rSp0032. This indicated that the two regions of rSp0032 likely interact when binding to specific sites on yeast. Given that non-challenged sea urchins show a preponderance of edited *Sp185/333* mRNAs<sup>[12, 16, 19]</sup> that are translated into truncated natSp185/333 proteins missing the his-rich region<sup>[15]</sup>, this suggests that the possible expanded binding characteristics of truncated gly-rich Sp185/333 proteins may have important immunological surveillance functions in the sea urchin.

rSp0032 Binds to  $\beta$ -1,3-glucan, Lipopolysaccharide and Flagellin, but not to Peptidoglycan

Based on the binding characteristics to bacteria and yeast and the observation of flagellin associated with rSp0032 on Western blots (Table 1), we tested whether PAMPs were the binding targets on foreign cells. Lipopolysaccharide (LPS from *E. coli*),  $\beta$ -1,3-glucan (from *S. cerevisiae*), peptidoglycan (PGN from *Bacillus subtilis*), and flagellin (from *Salmonella typhimurium*) were evaluated as binding



**Figure 2. The multitasking binding activities of rSp0032.** rSp0032 is shown as an extended, unfolded disordered protein, as predicted from the amino acid sequence. The gly-rich region is shown in orange, the his-rich region is blue (see Fig. 1A), and the C-terminal region is shown in gray. Red arrows indicate the targets to which rSp0032 binds including LPS from *E. coli*, flagellin from *Vibrio* and *Salmonella*, and β-1,3-glucan from *Saccharomyces*. The black line indicates that rSp0032 does not bind to *Bacillus* species, nor to PGN isolated from *B. subtilis*. Binding to *Vibrio* LPS is assumed from binding results to whole *Vibrio* cells. Similarly, lack of binding to *Bacillus* flagellin is assumed from binding results using whole *Bacillus* cells. This figure is modified from the graphical abstract published in association with Lun et al.<sup>[17]</sup>.

targets for rSp0032 by ELISA. rSp0032 demonstrated saturable binding to LPS with high affinity ( $K_d = 0.9 \mu\text{M}$ ) and slightly lower affinity for β-1,3-glucan ( $K_d = 3 \mu\text{M}$ ) and flagellin ( $K_d = 6.1 \mu\text{M}$ ) but did not bind to PGN. It was unusual for a single protein to bind to several PAMPs composed of different sugars in addition to a non-glycosylated protein. Consequently, when competitive ELISA was used to evaluate the specificity of rSp0032 binding to these PAMPs (Fig. 1B) results indicated that when rSp0032 was pre-mixed with increasing amounts of each PAMP and then evaluated for binding to LPS, binding was competed by LPS as expected. However, binding to LPS was also competed by flagellin and β-1,3-glucan but not by PGN. These results indicated that rSp0032 was capable of binding specifically to three very different PAMPs.

## Discussion

The recombinant Sp185/333 protein, rSp0032, exhibits specific and tight binding to Gram-negative bacteria and yeast, shows similar binding to LPS, β-1,3-glucan and flagellin, but fails to bind to Gram-positive bacteria and PGN, and we assume it fails to bind to flagellin from *Bacillus* (Fig. 2). When the rGly-rich and rHis-rich fragments are separated from rSp0032, the binding specificity is altered

for the rGly-rich fragment, which shows expanded binding compared to the full-length protein. The rC-Gly fragment appears to be entirely responsible for rSp0032 multimerization and may have similar functions for all versions of the natSp185/333 proteins. The rSp0032 binding study reported by Lun *et al.*<sup>[17]</sup> provides the first definitive evidence that rSp0032 is capable of binding a range of targets that include a subset of microbes and PAMPs. These multitasking activities are novel for an anti-pathogen protein and suggest that the large array of natSp185/333 proteins that are expressed *in vivo* by sea urchins<sup>[15, 16]</sup> may have a wide range of overlapping, anti-pathogen activities. Functioning together, these immune effector proteins may provide very effective host protection in sea urchins.

## rSp0032 Binds to Multiple PAMPs

The multitasking binding activities of rSp0032 to multiple PAMPs suggest molecular or structural similarities between LPS and β-1,3-glucan that are not shared with PGN to which rSp0032 does not bind. LPS and β-1,3-glucan are quite different, but both present possibilities for electrostatic interactions between the charged histidines in rSp0032 and modified sugars in the PAMPs including anionic phosphates on the glucosamine disaccharide of lipid A, the

phosphorylated polysaccharide core of LPS, and the non-modified hydroxyl groups on sugar components of both LPS and  $\beta$ -1,3-glucan. However, hydroxyl groups are also present on N acetyl glucosamine and N acetyl muramic acid in PGN suggesting that other factors, such as the number or accessibility of hydroxyl groups may also contribute to the binding specificity. Surprisingly, rSp0032 also binds to a non-glycosylated flagellin from *Salmonella typhimurium* and to flagellin from *V. diazotrophicus*. Flagellins are composed of four domains, of which two (D0 and D1) are positioned within the filament core, and the other two (D2 and D3) are hypervariable and exposed on the surface of the flagellum<sup>[20]</sup>. When the amino acid sequences of flagellins from *Vibrio*, *Salmonella*, *Escherichia* and *Bacillus* are aligned and compared, domain D2 is mostly missing from *B. subtilis* flagellin (because rSp0032 does not bind to *Bacillus*, we assume it does not bind to *Bacillus* flagellin) suggesting that this region may include the binding site for rSp0032. This is speculative at present and the question of how rSp0032 binds to flagellin, including versions that are not glycosylated, is not known.

#### rSp0032 may Function through Conformational Plasticity

The unusual binding activities of rSp0032 to a wide range of targets through non-covalent, charge-based interactions does not fit with strong and specific binding to microbes and PAMPs, and the inability to dissociate rSp0032 from *Vibrio* flagellin during the preparation for SDS-PAGE. Lun *et al.*<sup>[17]</sup> predicted a second phase of binding that may follow the initial charge-based interactions with conformational plasticity resulting in tight and non-reversible binding to targets. Given the range of targets, this would require significant variations in conformational changes to rSp0032, but *in silico* analysis of the amino acid sequence of rSp0032 (minus the leader) predicts intrinsic disorder and a protein composed entirely of extended loops without  $\alpha$  helices or  $\beta$  strands. If rSp0032 is intrinsically disordered, the second phase of rSp0032 binding may involve structural changes to folded order that is induced by the target and that may vary depending on the target. Flexible changes to the conformation of Sp0032 may be the basis for the strong affinity demonstrated by this anti-pathogen protein to very different target structures.

#### Conclusion

The application of the results reported by Lun *et al.*<sup>[17]</sup> to activities of the diverse natSp185/333 protein variants suggests that each variant may have a different but overlapping range of binding targets to which these multitasking proteins can bind. The binding mechanism may be a two-step process with initial electrostatic interactions

followed by conformational changes in the proteins that result in strong affinity binding. This may apply to all versions of the Sp185/333 proteins based on *in silico* evaluations indicating minimal secondary structures for all variations of these protein sequences. Consequently, the arrays of Sp185/333 proteins that are expressed by sea urchins<sup>[15, 16]</sup> may be interpreted in light of each variant having a range of distinct but overlapping binding activities for interactions with multiple foreign targets. If true, then it is unlikely that co-evolving microbes may be able to mount multiple simultaneous immune avoidance mechanisms to avoid the sea urchin innate immune response and will fail to increase virulence or to become pathogenic. The Sp185/333 proteins may be a major response system facilitating immune defenses in echinoids, which when taken into consideration with the long evolutionary history of these echinoderms, it fits with how well these animals are able to protect themselves from the wide variety of potential pathogens that are present in their near-short marine habitat.

#### Conflicting interests

The authors have declared that no conflict of interests exist.

#### Author Contributions

LCS and CML wrote the manuscript.

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#### Abbreviations

CF: coelomic fluid; C-Gly: C-terminal end of the gly-rich region; ELISA: enzyme-linked immunosorbent assay; FITC: fluorescein isothiocyanate; gly: glycine; his, histidine; kDa: kilo Daltons; LPS: lipopolysaccharide; MS/MS: tandem mass spectroscopy; natSp185/333: native Sp185/333 proteins; PAMP: pathogen associated molecular patterns; PGN: peptidoglycan.

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