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Shotgun proteomics of coelomic fluid from the purple sea urchin, *Strongylocentrotus purpuratus*

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ABSTRACT

The purple sea urchin has a complex immune system that is likely mediated by gene expression in coelomocytes (blood cells). A broad array of potential immune receptors and immune response proteins has been deduced from their gene models. Here we use shotgun mass spectrometry to describe 307 proteins with possible immune function in sea urchins including proteins involved in the complement pathway and numerous SRCRs. The relative abundance of dual oxidase 1, ceruloplasmin, ferritin and transferrin suggests the production of reactive oxygen species in coelomocytes and the sequestration of iron. Proteins such as selectin, cadherin, talin, galectin, amassin and the Von Willebrand factor may be involved in generating a strong clotting reaction. Cell signaling proteins include a guanine nucleotide binding protein, the Rho GDP dissociation factor, calcium storage molecules and a variety of lipoproteins. However, based on this dataset, the expression of TLRs, NLRs and fibrinogen domain containing proteins in coelomic fluid and coelomocytes could not be verified.

1. Introduction

The deuterostome lineage of the animal kingdom includes jawed vertebrates (gnathostomes), jawless vertebrates (agnathans), cephalochordates, urochordates, hemichordates and echinoderms. The last common ancestor of deuterostomes that lived in the precambrian (575 millions years ago) gave rise to echinoderms, of which sea urchins constitute a major taxon (class Echinodidea). Sea urchins are ubiquitously distributed in the world's oceans, constitute an important part of subtidal marine communities, and are an important fisheries resource. Sea urchins have been used as model organisms over the past century in developmental biology. The phylogenetic position of sea urchins and their importance in studies of embryonic development motivated sequencing the genome of the purple sea urchin (*Strongylocentrotus purpuratus*) (Sodergren et al., 2006; Tu et al., 2012). The sea urchin genome sequence confirmed the kinship between echinoderms and vertebrates and revealed a number of unexpected results. Perhaps the biggest surprise was the complexity of immune response genes in sea urchins (Hibino et al., 2006; Rast et al., 2006; Rast and Messier-Solek, 2008). Before publication of the genome sequence, only a few laboratories worldwide had studied the sea urchin immune system. These earlier studies revealed the presence of a complement system similar to the chordate alternative complement pathway (Gross et al., 1999; Smith et al., 1999, 2006), antibacterial molecules (Haug et al., 2002; Li et al., 2009) and the expression of a large array of scavenger receptor cystein rich proteins (SRCRs) (Pancer, 2000). They also demonstrated that coelomocytes are the main effector cells of the immune response in sea urchins (Smith et al., 2010). The genome sequence went onto show that the immune response genes of *S. purpuratus* incorporate an elaborate repertoire of innate pathogen recognition genes. These include 253 toll-like receptors genes (Buckley and Rast, 2012; Tu et al., 2012), of which the majority are closely related to those of vertebrates; 203 NACHT and leucine-rich repeat containing (NOD-like receptor, NLR) genes; 1095 SRCR domains distributed among 218 gene models; and 46 genes containing single fibrinogen domains that could potentially activate a lectin mediated complement pathway (Hibino et al., 2006). The genome also provided some evidence for the presence of pathogen specific hypervariable defense systems. One sea urchin gene cluster showed high similarity to RAG1/2 genes that are used in vertebrates to generate the molecular hypervariability of antibodies and T-cell receptors by V(D)J recombination (Fugmann et al., 2006; Hibino et al., 2006). Another gene family (the *Sp185/333* family) has all the hallmarks of a highly

Abbreviations: NSAF, normal spectral abundance factor; CF, coelomic fluid.

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variable immune response family (Nair et al., 2005; Terwilliger et al., 2006, 2007; Buckley and Smith, 2007; Dheilly et al., 2009, 2011a; reviewed in Smith, 2012).

Given the apparent complexity of the sea urchin immune system, it has become a priority to characterize the immunome of sea urchin coelomocytes and coelomic fluid to reconstruct the complex pathways that provide this organism with protection against pathogens. We report here a large scale (shotgun) proteomics analysis that provides an initial map of intracellular functions of *S. purpuratus* coelomocytes and activities of the coelomic fluid. Our approach employed digesting whole coelomic fluid protein extracts prior to fractionation by liquid chromatography and analysis by tandem mass spectrometry (LC–MS/MS) (Domon and Aebersold, 2006). This approach resulted in improved quantification and better coverage of the proteome compared to more traditional two-dimensional gel electrophoresis analysis.

2. Materials and methods

2.1. Sea urchins, immunological challenge and sample collection

Adult *S. purpuratus* were supplied by Marinus Scientific Inc. (Long Beach, CA) after collection from the coast of Southern California (USA). They were maintained in the laboratory for 6 months as described previously (Clow et al., 2000; Gross et al., 2000; Nair et al., 2005). *S. purpuratus* become immunoquiescent after long-term housing in the laboratory (greater than 8 months without significant disturbance) (3). Consequently, to ensure that the sea urchins were immunologically active before sample collection, they ($n = 3$ individuals) were challenged by injecting 2 μg of lipopolysaccharide (LPS, Sigma Aldrich, St. Louis, MO) per ml of coelomic fluid (CF), as previously described (Smith et al., 1995, 1996; Clow et al., 2000). Twenty-four hours after injection, a 23-gauge needle attached to a 1 ml syringe was inserted through the peristomeum into the coelomic cavity and CF was withdrawn without anticoagulant. The CF was immediately expelled into a 1 ml tube and mixed with 100 μl of urea sample buffer (2.4 M Tris–HCl pH 6.8; 0.25% SDS; 4 M urea; 20% glycerol). Samples were stored at −70 °C until used. The samples were freeze dried and the proteins extracted in SDS sample buffer (0.05 M Tris HCl, 10% glycerol, 10% SDS, 1% DTT). The total protein content of each sample was determined using Bradford reagent (Bio-Rad Laboratories Inc.).

2.2. One-dimensional electrophoresis (1DE)

CF proteins in SDS sample buffer (~10 μg per well) were separated on 7.5% Bis-Tris polyacrylamide gels at 180 V for 1 h. After electrophoresis, proteins were visualized using a standard Coomassie blue protocol. Coomassie blue stained gels were washed twice in water (10 min per wash) and individual lanes were cut into 16 slices of equal size. Proteins were reduced, alkylated and subjected to trypsin digestion as previously described (Dheilly et al., 2009).

2.3. Mass spectrometry and data analysis

Mass spectrometric analysis was performed at the Australian Proteome Analysis Facility (APAF; Macquarie University, North Ryde, New South Wales, Australia). The tryptic digest extracts from 1DE gel slices were subjected to data-dependent nanocapillary reversed phase liquid chromatography followed by electrospray ionization using a Thermo LCQ Deca ion trap mass spectrometer (Thermo; LC–MS/MS) as described previously (Dheilly et al., 2009).

2.4. Protein identification

Global Proteome Machine Organisation (GPM) open source software (www.thegpm.org) was used to search peptide sequences against a combined *Strongylocentrotus* database created with downloaded sequences from NCBI. This FASTA format database contained 44,037 protein sequences comprising all *Srongylocentrotus* sequences held by NCBI as of April 2008. The database also incorporated a list of common human and trypsin peptide contaminants. The search was made against all reversed sequences to evaluate the False Discovery Rate (FDR). Search parameters included MS and MS/MS tolerances of ±2 Da or ±0.2 Da, tolerance of up to 3 missed tryptic cleavages and K/R–P cleavages. Fixed modifications were set for carbamidomethylation of cysteine and variable modifications were set for oxidation of methionine. Raw data files are provided in Supplementary data 1. Only proteins (i) for which at least two unique peptides (Supplementary data 2) were identified, (ii) that were present in at least 2 of the 3 sea urchins that were analyzed (Supplementary data 2), and (iii) that yielded a $\log(e)$ value < -9 and a total of 4 spectral counts over the 3 samples were conserved within the analysis (Supplementary data 3). After filtering the sequences, no reversed sequences were identified indicating an FDR of <1%.

2.5. Statistical analysis

Normal Spectral Abundance Factor (NSAF) values were determined as previously described for each protein using the aquired data (Zybailov et al., 2007). Briefly, when a null spectral count was obtained for one of the samples, a fraction of 0.2 replaced the null result so that the analysis could be continued. For each sample i, the $NSAF_i$ of protein k was deemed to be the number of spectral counts (SpC) that identified protein k, divided by the molecular weight of the protein (MW, kDa). This was divided by the sum of (SpC/MW) for all N proteins in the experiments (Eq. 1)

$$NSAF_i = \frac{(SpC/MW)_k}{\sum_{i=1}^{N}(SpC/MW)} \quad (1)$$

For each protein k, the sum S of all spectral counts obtained from the 3 samples was calculated, which resulted in the corresponding $NSAF_S$ values.

3. Results and discussion

3.1. High reproducibility

The three sea urchins that provided the protein samples for this study were assumed to be immunoquiescent after having been kept for greater than 6 months without disturbance in a closed (recirculating) marine aquarium as described (Gross et al., 2000). Hence the animals were injected with LPS prior to sample collection. This has been shown previously to reverse immunoquiescence and return sea urchins to an immunologically active state (Pancer, 2000). We initially wished to study the proteome of cell free coelomic fluid and coelomocytes separately. However, a very strong clotting reaction is observed when we extract the coelomic fluid with or without anticoagulant. This clotting traps the most abundant and high molecular weight proteins when we separate the cells from the fluid by centrifugation. In addition, it is not possible to resuspend cells that have been pelleted because they stick tightly to each other. Thus, the following study investigates the proteome of the whole coelomic fluid. A total of 307 proteins were identified with great confidence in the coelomic fluid from the three immunologically activated sea urchins (FDR < 0.1%). The 307 proteins identified were among the most abundant proteins of the coelomic fluid of the three sea urchins and are therefore predicted to have important functions.

The reproducibility of the data was confirmed by plotting the log(NSAF) values of the samples from the three sea urchins against

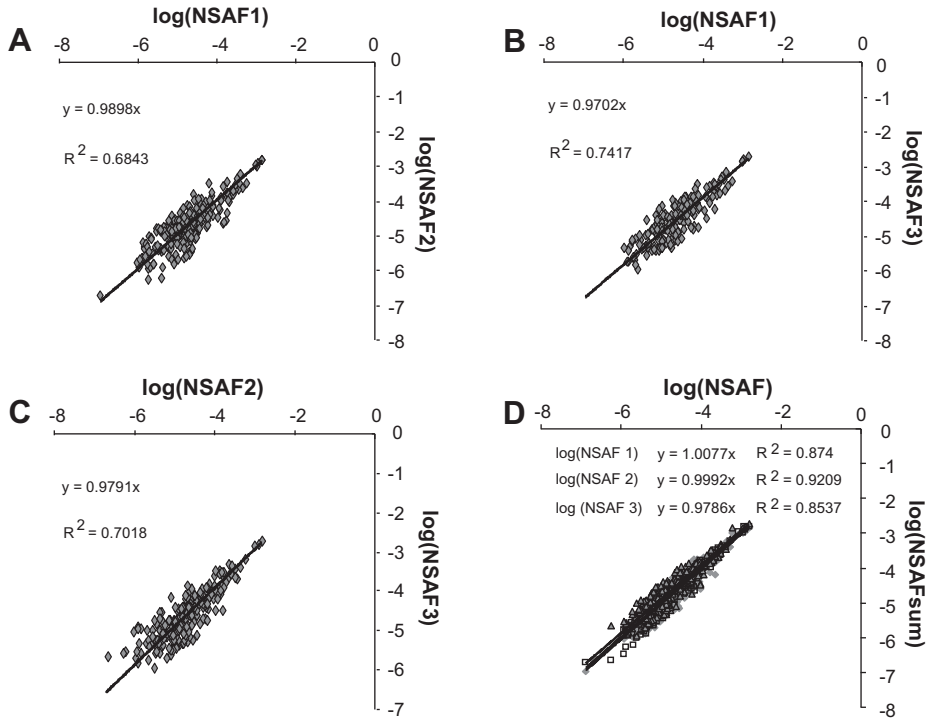

Fig. 1. Distribution of log(NSAF). The log(NSAF) of the samples from three sea urchins (1, 2, and 3) are plotted against each other. (A) log($NSAF_1$) vs. log($NSAF_2$); (B) log($NSAF_1$) vs. log($NSAF_3$); (C) log($NSAF_2$) vs. log($NSAF_3$); (D) the log(NSAF) values obtained from the three samples are plotted against log($NSAF_S$).

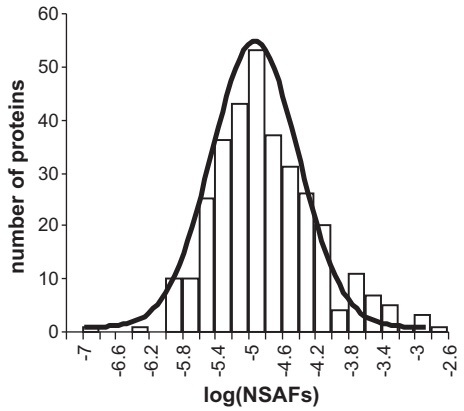

Fig. 2. Distribution of natural log of $NSAF_S$ The distribution fits a Gaussian curve with a mean of −4.78 and a standard deviation of 0.62.

each other (Fig. 1A–C). We obtained linear relationships with r^2 values ranging from 0.68 to 0.74 (Fig. 1), which indicated that the three samples were very similar. To determine whether the sum of the spectral counts obtained from the three sea urchin samples could be used for quantitative analysis, the log($NSAF_S$) values were plotted against the log(NSAF) values of the three samples (Fig. 1D). This yielded r^2 values ranging between 0.85 and 0.92, confirming that *S* was a good representation of the protein concentration in the pooled data of the three sea urchins. The natural log of the NSAFs values were plotted against the number of proteins obtained and showed a normal distribution with a mean of −4.78 and a standard deviation of 0.62 (Fig. 2).

3.2. Classification of proteins

Of the 307 proteins that were identified with more than two unique peptides, not all can be assumed to be unique (Table 2; Supplementary data 3). Many proteins had multiple homologs. These included actin, SRCRs, echinonectin, integrin, apolipoprotein B and heat shock proteins. However all matches were assigned to unique peptides suggesting that different homologues existed in sea

Table 1
Classification of the proteins identified from the coelomic fluid of challenged sea urchins.[a]

Functional groups	Proteins	Spectral counts	$NSAF_S$
Cell structure, shape and mobility	53	11915	9.3E−03
Cell signaling	48	2327	1.2E−03
Nucleic acid and protein metabolism and processing	37	673	9.1E−04
Immune response	33	3364	1.8E−03
Cell adhesion	28	1693	9.8E−04
Stress response, detoxification	27	3656	1.3E−03
Energy metabolism	20	472	3.7E−04
Lysosomes, proteases and peptidases	17	816	3.0E−04
Cell proliferation, reproduction and development	17	729	4.4E−04
Exchanger and ATPases	13	341	2.8E−04
Intracellular transport	6	179	1.1E−04
Others	3	142	9.1E−05
Unknown	6	105	1.0E−04

[a] The number of proteins identified per functional group is shown, as well as the sum of the spectral counts and the $NSAF_S$ values.

Table 2
Predicted protein function of the proteins identified from the coelomic fluid of challenged sea urchins and classification in 13 functional groups.

Accession number	Predicted protein function
Cell structure, shape and mobility	
gi|47551037|	cytoskeletal actin CyIIb
gi|115956638|	cytoskeletal actin CyIIb
gi|47550921|	cytoskeletal actin
gi|115949854|	cytoskeletal actin
gi|47551035|	cytoskeletal actin CyIIIb
gi|1703135|	cytoskeletal actin CyIIIb
gi|115943916|	actin
gi|115971461|	cytoplasmic actin
gi|115968818|	filamin-c isoform 4
gi|115964477|	actin-related protein 2 3 complex subunit 1a
gi|115958283|	actin-related protein 2 3 complex subunit 4
gi|47551153|	profilin
gi|115955758|	cofilin
gi|47551049|	fascin
gi|115948375|	capping protein (actin filament) muscle z- alpha 2
gi|115961398|	capping protein (actin filament) muscle z- beta
gi|115960968|	actin-related protein 3
gi|115944135|	arp2 actin-related protein 2 homolog
gi|115936071|	actin-related protein 2 3 complex subunit 3
gi|115961140|	gelsolin protein 2-like
gi|115930179|	gelsolin
gi|115933962|	gelsolin protein 2-like
gi|115971193|	rho small gtp binding protein rac1
gi|115939472|	CDC42, cell division cycle 42
gi|115974358|	adp-ribosylation factor 4
gi|115939656|	adp-ribosylation factor 2
gi|115976308|	Lymphocyte cytosolic protein 1, 65-kda macrophage
gi|115945231|	nck-associated protein 1
gi|115945717|	Enabled homolog
gi|115630732|	myosin, heavy polypeptide 10, non-muscle
gi|115960644|	PREDICTED: similar to myosin heavy chain
gi|115925414|	myosin
gi|115963096|	myosin heavy non-muscle-like
gi|115977085|	PREDICTED: similar to alpha-1 tubulin
gi|115960585|	tubulin, alpha 2 isoform 1
gi|115971215|	tubulin
gi|115950170|	long microtubule-associated protein 1A; long MAP1A
gi|115975440|	moesin
gi|115974666|	coronin
gi|115939091|	lethal giant larvae protein homolog 2 isoform 1
gi|115929203|	alpha- sarcomeric-like isoform 1
gi|115955651|	short-chain collagen c4-like
gi|115896589|	short-chain collagen c4-like
gi|115945063|	fibrosurfin, partial
gi|115951109|	tensilin
gi|115974054|	fibropellin Ia
gi|115940889|	fibropellin-1- partial
gi|166795321|	advillin
gi|115974031|	reticulon 4 isoform B2
gi|115925936|	drebrin-like protein
gi|115931813|	lim and sh3 domain protein 1-like
gi|115948973|	PREDICTED: similar to CG8253-PA
gi|47550983|	nuclear intermediate filament protein
Cell adhesion	
gi|47551105|	integrin beta L subunit
gi|47551115|	integrin beta G subunit
gi|47551111|	integrin beta-C subunit
gi|115949077|	integrin alpha 7
gi|115910910|	fibronectin isoform 1
gi|115924509|	calpain B
gi|115950639|	vinculin-like isoform 1
gi|115906323|	vinculin family protein
gi|115960842|	talin-2
gi|115975224|	PREDICTED: similar to G-cadherin
gi|115720465|	PREDICTED: similar to selectin-like protein, partial
gi|47551235|	amassin
gi|118601062|	amassin-2
gi|118601054|	amassin-3
gi|118601058|	amassin 4
gi|115963192|	annexin a4
gi|115963196|	annexin a5
gi|115970125|	annexin a7
gi|115944296|	Galectin

Table 2 (*continued*)

Accession number	Predicted protein function
gi|115970015|	apolipoprotein b-100-like
gi|115851666|	Von willebrand factor
gi|115663188|	tetraspanin
gi|115956497|	tetraspanin family protein
gi|115945577|	transforming growth factor-beta-induced protein ig-h3
gi|115967683|	neural cell adhesion molecule 2
gi|115967917|	probable g-protein coupled receptor 128-like
gi|115953252|	foot protein-4 variant-2
gi|115975625|	plexin- partial
Immune response	
gi|47551023|	complement component C3
gi|115618101|	complement component C3, partial
gi|47551047|	complement factor B
gi|47825406|	complement related-long precursor
gi|115960292|	complement related-long precursor, partial
gi|115974487|	complement receptor type 2- partial
gi|115970612|	complement receptor type 2- partial
gi|115970610|	complement receptor type 2- partial
gi|115971048|	complement receptor type 2- partial
gi|115934253|	scavenger receptor cysteine-rich protein
gi|47551161|	scavenger receptor cysteine-rich protein
gi|47550953|	scavenger receptor cysteine-rich protein
gi|47551167|	scavenger receptor cysteine-rich protein type 5
gi|47550951|	scavenger receptor cysteine-rich protein variant 2
gi|47551157|	scavenger receptor cysteine-rich protein type 12
gi|115968904|	scavenger receptor cysteine-rich protein type 12 precursor
gi|115936114|	scavenger receptor cysteine-rich protein precursor, partial
gi|115949177|	scavenger receptor cysteine-rich protein type 12 precursor, partial
gi|115950182|	scavenger receptor cysteine-rich protein type 12 precursor
gi|115968906|	scavenger receptor cysteine-rich protein type 12 precursor
gi|115973483|	scavenger receptor cysteine-rich protein type 12 precursor, partial
gi|115975788|	scavenger receptor cysteine-rich protein
gi|115944063|	PREDICTED: hypothetical protein (srcr domains)
gi|118421783|	Sp185/333
gi|47550955|	DD104 protein, upregulated upon bacterial challenge and trauma
gi|115619038|	arylsulfatase a
gi|115950487|	arylsulfatase a
gi|115968659|	PREDICTED: similar to rhamnose-binding lectin (SAL), partial
gi|115971190|	mannan-binding lectin serine protease 1
gi|115940254|	cyclophilin
gi|115940188|	cyclophilin
gi|115966254|	sam domain and hd domain-containing protein 1-like
gi|115958371|	lysozyme
Lysosomes, proteases and peptidases	
gi|115938941|	lysosome-associated membrane glycoprotein 1-like
gi|115955224|	cathepsin z precursor
gi|115959123|	Thrombin
gi|115958627|	thrombin
gi|115975288|	Thrombospondin
gi|115953033|	Aminopeptidase puromycin sensitive
gi|115971224|	aminopeptidase
gi|115940918|	aminopeptidase like 1
gi|115925688|	CNDP dipeptidase 2 (metallopeptidase M20 family)
gi|115774731|	leukotriene A4 hydrolase
gi|115955254|	alpha macroglobulin
gi|115786699|	alpha-2-macroglobulin, partial
gi|115949647|	alpha macroglobulin, partial
gi|115924999|	tissue inhibitor of metalloproteinase TIMP
gi|115925007|	tissue inhibitor of metalloproteinase TIMP
gi|115769879|	c3 and pzp-like alpha-2-macroglobulin domain-containing protein 8-like
gi|115970724|	Valosin containing protein
Intracellular transport	
gi|115963658|	major vault protein
gi|115950948|	Adaptor-related protein complex 2, beta 1 subunit, partial
gi|115968951|	adaptor protein complex AP2
gi|115936009|	flotillin-1-like isoform 1
gi|115928607|	flotilin
gi|115931510|	sorting nexin-2
Exchangers and ATPases	
gi|115941346|	v-type proton atpase catalytic subunit a
gi|115935979|	voltage-dependent anion channel 2
gi|115966189|	mitochondrial H(+)-transporting ATPase beta subunit
gi|115710920|	Na+/K + transporting ATPase alpha subunit

(*continued on next page*)

Table 2 (*continued*)

Accession number	Predicted protein function
gi|115926327|	sodium calcium exchanger 3
gi|115926329|	sodium calcium exchanger 3
gi|115924511|	myosin + atp binding cassette ABC containing
gi|47551121|	mitochondrial ATP synthase alpha subunit precursor
gi|115926312|	H(+) transporting ATP synthase
gi|115697801|	Nicotinamide nucleotide transhydrogenase
gi|115956824|	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4
gi|115939138|	solute carrier family 25 (mitochondrial carrier phosphate carrier) member 3
gi|115940494|	solute carrier family 25 (mitochondrial aralar) member 12
Cell signaling	
gi|47551041|	ER calcistorin
gi|42794334|	phospholipase C delta isoform
gi|115954188|	Sorcin
gi|115968621|	Calponin
gi|115973453|	Calponin
gi|115976312|	Calponin
gi|115943069|	Calponin
gi|115945701|	Calcium-binding protein p22
gi|47825400|	guanine nucleotide-binding protein G(i) alpha subunit
gi|47825404|	guanine nucleotide-binding protein G(12) alpha subunit
gi|115936803|	heterotrimeric guanine nucleotide-binding protein beta subunit isoform 3
gi|115968932|	Arrestin N superfamily
gi|115976839|	GDP-dissociation inhibitor
gi|115973265|	rho GDP dissociation inhibitor
gi|115945943|	GDP dissociation inhibitor
gi|115964065|	adenylyl cyclase-associated protein 1
gi|115964067|	adenylyl cyclase associated protein 1
gi|115945715|	Ras-related protein ORAB-1
gi|115966293|	ras-related protein rab-8a
gi|115974451|	ras-related protein rab-10
gi|115949757|	ras-related protein rap-1b precursor
gi|115974362|	ras-related protein rab-7a
gi|115975006|	ras-related protein rab-2a
gi|115938855|	ras-related protein rab-11b
gi|115940967|	Dynamin 2, partial
gi|115934007|	TRAF4-associated factor 2
gi|115927899|	programmed cell death 8. apoptosis inducing factor
gi|115894557|	RACK
gi|115936246|	serine/threonine protein kinase
gi|115973105|	pyruvate kinase
gi|115968426|	arginine kinase
gi|115935133|	adenylate kinase 2 isoform 2
gi|47551197|	src-family protein tyrosine kinase
gi|115929326|	ceramide kinase
gi|115929815|	Nucleoside diphosphate kinase family protein
gi|115954217|	hexokinase I
gi|115958629|	low-density lipoprotein receptor-related protein 4
gi|115685173|	low-density lipoprotein receptor-related protein 6 isoform 2
gi|115966000|	low density lipoprotein receptor-related protein 6
gi|115940258|	lipid raft asssocitaed protein 2
gi|115944489|	esterase lipase
gi|115976887|	steroidogenic acute regulatory mitochondrial
gi|115937346|	apolipophorin precursor protein
gi|115940166|	apolipoprotein B
gi|115970013|	apolipoprotein B-100-like
gi|115926610|	proprotein convertase subtilisin/kexin type 9 preproprotein
gi|115940411|	14–3-3-like protein 2
gi|160623362|	putative 14–3-3 epsilon isoform
Stress response, detoxification	
gi|115972586|	71 Kd heat shock cognate protein
gi|115958481|	70 kDa heat shock protein precursor
gi|115893581|	70 kDa heat shock protein protein
gi|115972590|	heat shock protein 70 isoform 1
gi|115891388|	heat shock 90 kDa protein, partial
gi|115964822|	mitochondrial chaperonin Hsp56
gi|115924889|	t-complex protein 1 subunit epsilon
gi|115954867|	t-complex protein 1 subunit delta
gi|115930256|	t-complex protein 1 subunit gamma
gi|115815329|	t-complex protein 1 subunit zeta isoform 2
gi|115956641|	t-complex protein 1 subunit 7
gi|115944450|	t-complex protein 1 subunit theta-like
gi|115944169|	dual oxidase 1 isoform 1
gi|115944173|	dual oxidase maturation factor
gi|115945316|	Choline dehydrogenase

Table 2 (*continued*)

Accession number	Predicted protein function
gi|115661720|	sucinate semialdehyde dehydrogenase
gi|115926010|	glutathione peroxidase, partial
gi|115937420|	catalase
gi|115959568|	glutathione reductase
gi|115685450|	phosphogluconate dehydrogenase
gi|115926884|	carbonic anhydrase alpha
gi|115968538|	ferritin
gi|115963947|	ceruloplasmin
gi|115926107|	ferroxidase
gi|115963949|	ferroxidase
gi|115970375|	melanotransferrin/EOS47
gi|47551123|	major yolk protein
Energy metabolism	
gi|115959412|	fructose-biphosphate aldolase
gi|115955959|	glucosamine-6-phosphate isomerase 1
gi|115929324|	glucose-6-phosphate 1-dehydrogenase
gi|115972829|	phosphoglucose isomerase
gi|115738231|	glyceraldehyde-3-phosphate dehydrogenase
gi|115961332|	glutamate dehydrogenase 1
gi|115680328|	cytosolic malate dehydrogenase
gi|115939485|	malate dehydrogenase
gi|115931669|	aldehyde dehydrogenase 1A2 isoform 1
gi|115968074|	aldehyde dehydrogenase
gi|115963910|	flavonol reductase cinnamoyl-CoA reductase
gi|115958742|	lactate dehydrogenase
gi|115926187|	Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A
gi|115978426|	dihydrolipoyllysin S succinyltransferase
gi|115970392|	independent phosphoglycerate mutase
gi|115964543|	transaldolase
gi|115944251|	transketolase isoform 1
gi|115932265|	aspartate aminotransferase cytoplasmic
gi|115924009|	Methylthioadenosine phosphorylase
gi|115945027|	integral membrane protein 1 oligosaccharyltransferase complex
Nucleic acid and protein metabolism and processing	
gi|47551089|	histone H1-beta
gi|47551095|	histone H1-delta
gi|47551091|	late histone H1-gamma
gi|62177162|	histone H2A
gi|115718598|	Histone H2AV (H2A.F/Z)
gi|47551075|	late histone L1 H2b
gi|47551065|	histone H3
gi|47551061|	H4 histone protein
gi|115955282|	heterogenous nuclear ribonucleoprotein K
gi|148539566|	eukaryotic initiation factor 4a
gi|115970523|	elongation factor 1 alpha isoform 2
gi|115924286|	translationally controlled tumor protein isoform 2
gi|115911567|	aspartyl tRNA synthase
gi|115652043|	histidyl-tRNA synthetase, partial
gi|115970083|	ubiquitin/40S ribosomal protein S27a
gi|115934350|	ubiquitin-activating enzyme E1
gi|115958412|	Ribosomal protein 40S
gi|115933954|	ribosomal protein L13, partial
gi|115954953|	ribosomal protein L28
gi|115957127|	ribosomal protein L5
gi|115928298|	40S ribosomal protein S24
gi|115649138|	ribosomal protein S3
gi|115921067|	ribosomal protein L23a
gi|115959136|	ribosomal protein S8e
gi|115940610|	Ribosomal protein
gi|115974576|	ribosomal protein S14e
gi|115974148|	Ribophorin I
gi|115975522|	Ribophorin II
gi|115950386|	transport protein sec61 alpha
gi|115926828|	eukaryotic translation elongation factor isoform 2
gi|115940461|	protein disulfate isomerase
gi|115966085|	endo-beta-N-acetylglucosaminidase, partial
gi|115974434|	adenosylhomocysteinase
gi|115973107|	NADH cytochrome reductase
gi|115946250|	serine mitochondrial isoform 3
gi|115929116|	aminoimidazole-4-carboxamide ribonucleotidetransformylase/IMP cyclohydrolase (Inosine monophosphate synthase)
gi|115940855|	actin-interacting protein 1-like isoform 1
Cell proliferation, reproduction and development	
gi|115960970|	proliferation-associated protein 1
gi|115948485|	mollusk-derived growth factor; MDGF, partial

(*continued on next page*)

Table 2 (*continued*)

Accession number	Predicted protein function
gi|115968740|	Septin 6
gi|115945705|	Testilin
gi|115770276|	zonadhesin
gi|115957151|	vitellogenin
gi|115956777|	ECM 18
gi|115945319|	alkaline phosphatase
gi|115964625|	nmrA like family domain containing
gi|115956476|	apextrin
gi|115955360|	apextrin
gi|115924200|	secreted lectin homolog; HeEL-1
gi|115620061|	echinonectin
gi|115951163|	echinonectin
gi|115973518|	echinonectin
gi|115899605|	echinonectin
gi|115952980|	echinonectin
Others	
gi|115955764|	Cry5 protein
gi|115976552|	receptor accessory protein 5
gi|115925590|	mucin 5, partial
Unknown	
gi|115964419|	PREDICTED: hypothetical protein
gi|115930346|	PREDICTED: similar to conserved hypothetical protein
gi|115967751|	PREDICTED: hypothetical protein
gi|115969396|	PREDICTED: hypothetical protein
gi|115937367|	PREDICTED: hypothetical protein, partial
gi|115967840|	PREDICTED: similar to 0910001A06Rik protein isoform 2

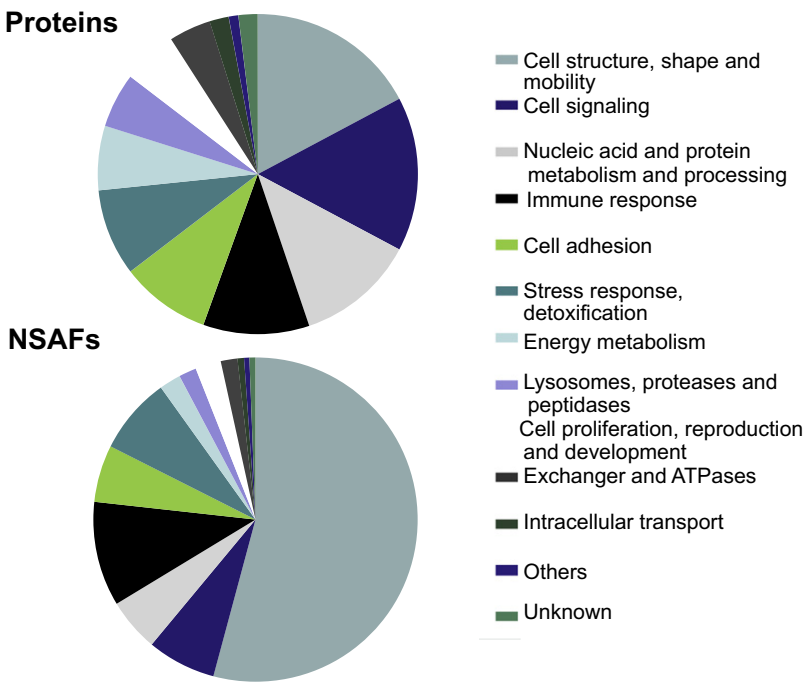

Fig. 3. Classification of the proteins identified from the coelomic fluid of *S. purpuratus*. The quantification was estimated by the normal spectral abundance factor of the sum *S* ($NSAF_S$) obtained from the three individual sea urchins for each protein *k* ($n = 307$, see Supplementary data 3).

urchins and were expressed in the coelomic fluid. These proteins were retained within the following analyses.

The proteins were grouped into 13 functional categories based on information generated by the *S. purpuratus* genome sequencing project (Sodergren et al., 2006), the existing database (NCBI), and a complementary annotation performed with blast searches, gene ontology term annotation and conserved domain searches (Table 2; Supplementary data 3). These were cell structure, shape and mobility; cell adhesion; immune response; lysosomes, proteases and peptidases; intracellular transport; exchangers and ATPases; cell signaling; stress response and detoxification; energy metabolism; nucleic acid and protein metabolism and processing; and cell proliferation, reproduction and development (Fig. 3). Three proteins could not be classified into any of these categories and were grouped as "others". There were a further six proteins that did not show any similarity to known proteins or that matched unknown proteins. These were grouped as "unknowns". It is noteworthy that 267 of the 307 proteins identified were PREDICTED proteins

derived from the genome of *S. purpuratus*. Hence, we can confirm for the first time that these proteins are expressed by (and are present in) *S. purpuratus* coelomic fluid.

An estimation of the quantity of proteins was provided using the NSAFs values. These values enabled a comparison of the abundance of each functional category within the proteome (Fig. 3A). Briefly, no correlation was found between the number of proteins identified within a functional category and the abundance of the proteins within sea urchin coelomic fluid. With 53 and 48 proteins identified respectively, the categories cell structure, shape and mobility and cell signaling were the most highly represented within the samples. However, according to the NSAFs, proteins within the categories cell structure, shape and mobility and immune response were the most abundant. Except for the category of biological process, the great majority of the identified proteins may be related to immunity. For example, it is reasonable to assume that the lipid transport proteins identified in abundance may be involved in regulating the lipid metabolism. However, they may also have an immune-related function. In the following discussion, we focus on the potential immune-related function of the proteins within each category from the most represented to the least represented within each sample (Table 1; Fig. 3).

4. Protein categories

4.1. Cell structure, shape and mobility

Proteins involved in cell structure, shape and mobility were the most common with 53 proteins identified (Fig. 3; Table 1; Supplementary data 3). The most abundant of all proteins was actin, reflecting the extensively malleable cytoskeleton of phagocytic coelomocytes (Edds, 1977, 1980; Henson et al., 2003). Other cytoskeletal proteins with high NSAFs values included profilin, fascin, cofilin, gelsolin, coronin, mysosin, tubulin, actinin, collagen and a protein similar to an Enabled homolog (EnaH) from *Drosophila melanogaster*. All of these proteins are involved in the dynamic properties of actin filaments and cytoskeletal moditifications in a broad range of species. EnaH is a member of the Ena/VASP family and couples F actin to the cell surface (Kuhnel et al., 2004), while capzb-prov protein caps F actin, and gelsolin and cofilin sever actin filaments (Southwick, 2000). Cofilin promotes the dissociation of actin monomers from the pointed ends of the actin filaments and regulates the association of actin monomers at the barbed end of the filaments (Southwick, 2000). α-Actinin and fascin crosslink actin filaments into bundles (Tseng et al., 2001, 2002), whereas profilin recycles actin monomers (Blanchoin and Pollard, 1998). Coronin is localized to lamelipodia and has been implicated in phagocytosis and cell locomotion in the amphibian *Xenopus laevis* (Mishima and Nishida, 1999). Coronin may also be involved in the regulation of NADPH oxidase assembly on the phagolysosomes of mammalian neutrophils (Grogan et al., 1997).

The abundance of cytoskeletal and motility proteins that function in modulating the shape and structure of the actin cytoskeleton in coelomocytes suggests that these cells are involved in dynamic responses to their environment. These responses are likely to include modulating lamelipodia to filopodia and visa versa, extending filopodia as an initial step for phagocytosis, and amoeboid movement. Phagocytes can represent up to 82% of the coelomocyte population (Gross et al., 2000; Smith et al., 2006, 2010). They are highly mobile cells that can quickly develop extended filopodia and lamelipodia (Edds et al., 1983), as reflected by the preponderance of cytoskeletal proteins identified in the coelomic fluid proteome and transcriptome (Smith et al., 1996; Nair et al., 2005; Dheilly et al., 2012).

4.2. Intracellular signaling

A wide variety of proteins involved in signaling pathways were identified (48 proteins) (Fig. 3; Table 1). However, their total number of spectral counts was low and only a few of these proteins were abundant (Supplementary data 3). Homologues of calcium binding proteins and proteins involved in GDP-based signaling systems were the most commonly identified proteins in this category. Calponin, calcium binding protein p22, and calcistorin all bind calcium, which is an important second messenger that functions in a variety of signaling pathways (Berridge et al., 2000; Berridge, 2006). More specifically, calcistorin (also called protein disulfide isomerase, PDI), is a calcium storage molecule that modulates integrin-dependent cell adhesiveness at sites of injury (Lebeche and Kaminer, 1992; Essex et al., 1995; Lahav et al., 2000). In vertebrates, inhibiting calcistorin also inhibits blood clot formation (Jasuja et al., 2012). One of the cDNAs identified in a previous EST analysis of immune challenged coelomocytes by Nair et al. (2005) encoded an ER calcistorin/PDI, suggesting that coelomocytes may increase the production and processing of proteins in response to immunological challenge. The guanine nucleotide binding protein is one of the most important in a group of signaling proteins that regulate immune function by regulating the intracellular concentrations of ions, sugars and cAMP (De vries et al., 2000). Rho GDP dissociation inhibitor plays an important role in oxidative responses within phagocytes by activating superoxide dismutase (Ridley, 2001). Adenylate cyclase associated protein regulates actin remodeling in response to cellular signaling and might be involved in endocytosis. The diversity of proteins within the signaling category suggests that coelomocytes have an extensive capacity to modulate intracellular activities. In animals, homologues of proteins with signaling functions have roles in regulating cellular immune responses, including oxidative killing mechanisms and the induction of antimicrobial activity.

Lipoproteins that are often involved in signaling were also found in abundance within the three sea urchin samples. We identified proteins with high similarities to apolipoprotein B and to low density lipoprotein receptor-related proteins 4 (Fig. 3; Table 1, Supplementary data 3). Apolipoprotein B is an apolipophorin precursor of the large lipid transfer protein superfamily and forms low density lipoprotein particles. The low density lipoprotein receptors are cell surface receptors involved in capturing a large diversity of low density lipoproteins and in the transmission of intracellular and extracellular signals (Gotthardt et al., 2000). The identification of these proteins in our samples reinforces the importance of the lipid metabolism in coelomocytes. Recent findings also suggest that molecules involved in lipid metabolism are often recruited to develop immunological responses (Miller et al., 2003; Van den Elzen et al., 2005). Most specifically, apolipophorins are involved in insect immune activation (Kim et al., 2004). Their abundance in *S. purpuratus* coelomic fluid implies a similar function in sea urchins.

4.3. Nucleic acid and protein metabolism and processing

Proteins involved in nucleic acid and protein metabolism were identified often (37 proteins) but their low NSAF values indicated low abundance (Fig. 3; Table 1; Supplementary data 3). Histones and ribosomal proteins constituted the most abundant matches although proteins involved in protein synthesis were also identified, and included two elongation factors. Recently, the role of extrachromosomal histones in immune responses has been demonstrated in both vertebrates and invertebrates. In humans, histone H2B mediates innate antiviral immune responses (Kouji et al., 2010) and histones H1 and H4 have antimicrobial properties (Wang et al., 2002; Lee et al., 2009). The antibacterial properties of histones

H1, H2A, H2B and H4 have been studied in numerous species including scallop (Li et al., 2007), oyster (Seo et al., 2011; Villamil and Gómez-chiarri, 2011) and salmon (Patrzykat et al., 2001). In echinoderms, an antimicrobial peptide derived from histone H2A has been identified in the sea star *Asterias rubens* (Maltseva et al., 2004). Interestingly, we found various histone proteins in *S. purpuratus* coelomic fluid and histone H4 was the most abundant protein within the category of nucleic acid and protein metabolism and processing (Supplementary data 3). Most interestingly, histone H2A was differentially expressed in response to challenge with lipopolysaccharide in the sea urchin *Heliocidaris erythrogramma* (Dheilly et al., 2012). Although the antibacterial activity of sea urchin histones has not yet been investigated, their abundance within the coelomic fluid (among the 307 most highly expressed proteins) and their conserved role in most metazoans suggests that they may be involved in the sea urchin innate immune response.

4.4. Immune response

We identified a total of 33 proteins homologous to molecules involved in immune responses (Fig. 3; Table 1; Supplementary data 3). The most abundant proteins in this category were complement component SpC3, a complement related protein, and complement factor SpBf. A basic complement system has already been reported in sea urchins, based on the identification of cDNAs encoding SpC3 and SpBf. These are the central proteins in one or more of the three known complement activation pathways: the classical, lectin and alternative pathways (Smith et al., 2006). In sea urchins, SpC3 has been shown to act as an opsonin (Clow et al., 2004), which is also an important aspect of its function in mammals. In addition to SpC3 and SpBf, we identified a protein similar to complement component C3 (49 amino acid substitutions in a sequence overlap of 222 amino acids), a complement related long precursor that shows similarities to factor H, factor I and complement components C6 and C7 (composed of 16 complement control domains, a galactose-bd-like domain and a kazal-type domain) (Multerer and Smith, 2004). Complement receptor type 2 (CR2) was also found. In vertebrates, CR2 interacts with bound C3 complement fragments C3d, C3dg and iC3b bound to the pathogen surface and initiates an immune response via the activation of antigen-specific B cells (Caroll, 1998). The abundance of numerous complement proteins and complement related proteins in the coelomic fluid suggests that coelomocytes are key mediators of immunological defense in sea urchins and that the integrated complement system of sea urchins may be more extensive than previously thought.

Various pathogen recognition molecules and a complement activator were found in abundance in the coelomic fluid of *S. purpuratus*. Indeed, rhamnose binding lectin recognizes globotriaosyl ceramide (Gb3) on the surface of macrophages, lipopolysaccharide on Gram-negative bacteria, lipoteichoic acid on Gram-positive bacteria, and glycolipids or glycoproteins from pathogens (reviewed in (Ogawa et al., 2011). Galectin (category cell adhesion) may recognize a wide variety of ligands including bacteria, algae and eukaryotic pathogens (Ogawa et al., 2011). Lysozyme, an enzyme abundant in most vertebrates body fluids, lyses most Gram-positive bacteria and a few Gram-negative and interact either directly or indirectly to modulate the complement activation cascade (Ogundele, 1998). In association with a serine protease such as the mannose-binding lectin serine protease 1 (MASP1), lectins may also lead to phagocytosis of the opsonized target via activation of the lectin complement pathway. The abundance of lectins, lysozyme and MASP1 further confirms the central role of the complement pathway in sea urchin innate immune response.

A large variety of SRCR proteins were also identified (Supplementary data 3). SRCR proteins constitute a family of highly variable cell surface and secreted proteins involved in the recognition of pathogens, the development of the immune system and regulation of immune responses (Yamada et al., 1998). In mammalians, members of the SRCR family are present on the surface of macrophages, as well as B and T cells (Yamada et al., 1998) and are also found as soluble proteins. Both the soluble and membrane bound SRCRs bind bacteria and low density lipoproteins and play an important role in innate immunity. We obtained a total of 14 matches for SRCR proteins reflecting the great variability of this family and its expression in sea urchin coelomic fluid (Fig. 4). Of the 14 SRCR matches that were obtained, six corresponded to SRCRs that had been identified previously; SRCR1, SRCR5, SRCR7 variant 2, SRCR12 and SRCR20 (Pancer, 2000) (Fig. 4). The remaining eight SRCR proteins that we identified had different structures to previously characterized forms, suggesting that they may have novel functions in coelomic fluid. Our results also confirmed that different patterns of SRCR proteins were expressed in individual sea urchins (Pancer, 2000).

The Sp185/333 protein family is also involved in sea urchin immunodefence. These proteins show significant sequence diversity and are expressed by two subsets of coelomocytes in the coelomic fluid and in intestinal tissue (Dheilly et al., 2011a; Brockton et al., 2008). Previous screening of an immune activated coelomoctye cDNA library showed that up to 73% of randomly chosen clones encoded Sp185/333 proteins (Nair et al., 2005). Surprisingly, the Sp185/333 proteins were present in only low abundance in our samples. Members of the Sp185/333 proteins family are overall very similar. They differ based on their mosaic block composition and single amino acid substitutions (Nair et al., 2005; Terwilliger et al., 2006, 2007; Buckley and Smith, 2007; Dheilly et al., 2009). The origin of the variability among these proteins does not enable differentiation among the different classes of Sp185/333 proteins by mass spectrometry analysis (Dheilly et al., 2009). Even though the function(s) of these proteins are unknown, it is noteworthy that they present an individual-specific heterogenous pattern of transcripts (Dheilly et al., 2009).

Two isoforms of arylsulafatase were highly abundant in the coelomic fluid, as was cyclophilin. Both arylsulfatase and cyclophilin have been identified previously by EST analysis (Smith et al., 1996). In mammals, arylsulfatase has a potential role in the cytotoxic activity of NK cells (Zucker-Franklin et al., 1983). Arylsulfatase is also expressed in the spherule cells of the sea cucumber, *Holoturia polii*, where its expression is enhanced during phagocytosis (Canicatti, 1988). In vertebrates, cyclophilins regulate immunosuppression by inhibiting calcineurin, which is a calcium and calmodulin dependent phosphatase involved in the activation of T lymphocytes and macrophages, lymphocyte degranulation, and apoptosis (Durette et al., 1988; Liu et al., 1991; Rusnak and Mertz, 2000). Furthermore, cyclophilins play a key role in the life cycle of viruses (Watashi and Shimotohno, 2007). A sam and hd domain containing protein 1 (SAMHD1) was also identified in the coelomic fluid of two of the three sea urchins. In humans, this enzyme depletes the pools of nucleotides available for reverse transcriptase activity and thus prevents viral replication (Lahouassa et al., 2012). In the future, the roles of these three molecules should be studied further because they may have significant implications for coelomocyte immune function.

4.5. Cell adhesion

A total of 28 proteins matched to molecules that are involved in cell adhesion (Fig. 3; Table 1). Among these, amassin variants were all highly abundant proteins in coelomic fluid (Supplementary data 3). These olfactomedin domain containing proteins are involved in intercoelomocyte adhesion during clotting (Hillier and Vacquier, 2003). Sea urchin amassins form disulfide-bonded complexes that

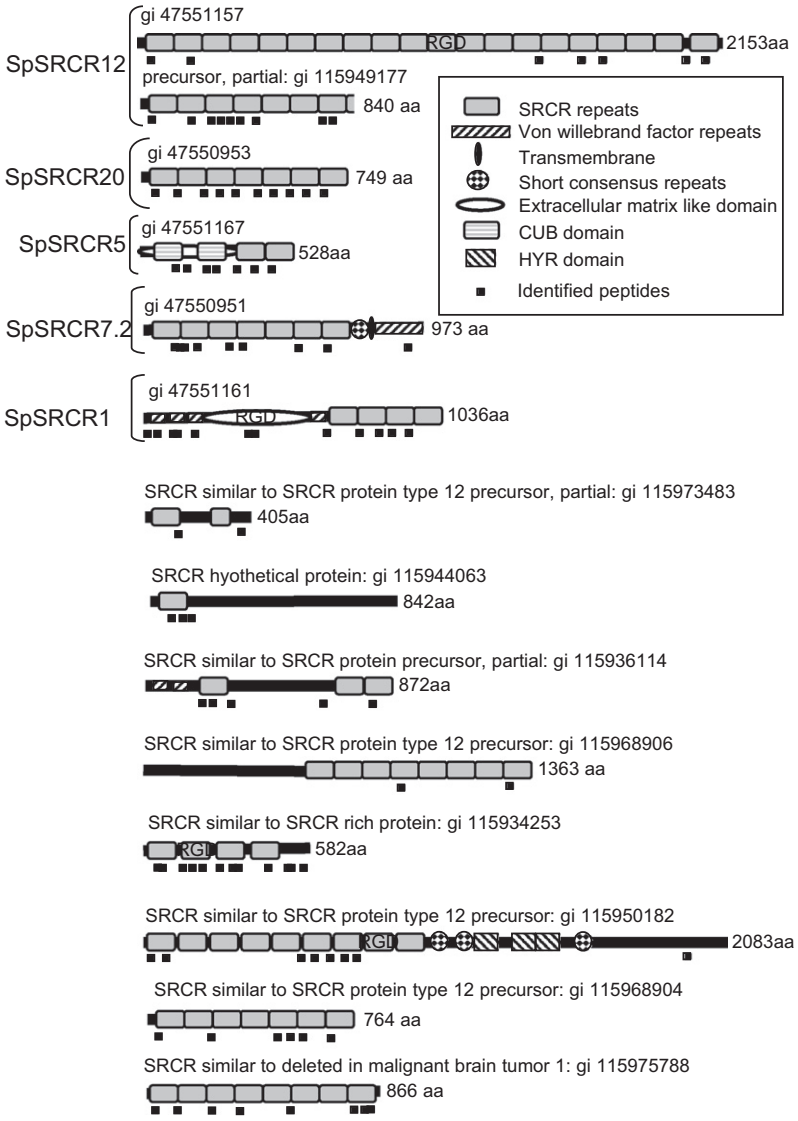

Fig. 4. Structure of the 14 sea urchin SRCRs identified in *S. purpuratus* coelomic fluid. Six SRCRs had been described previously, SpSRCR12, SpSRCR20, SpSRCR5, SpSRCR7.2 ans SpSRCR1. Eight new SRCRs were identified in *S. purpuratus* coelomic fluid. SRCR domains were found associated with Von Willebrand factor repeats, transmembrane domains, short consensus repeats, extracellular matrix like domains, CUB domains and HYR domains. The localization of the peptides identified in our samples and used to characterize the different SRCRs is indicated.

bind coelomocytes to each other forming large cellular aggregates that are thought to be involved with blocking coelomic fluid leakage from wounds, but might also be involved in sequestering bacteria and other pathogens at wound sites. Previous studies have shown that expression of amassin in sea urchins is enhanced after the injection of LPS (Nair et al., 2005; Dheilly et al., 2012).

Other putative cell adhesion proteins that were abundant in our samples included Von Willebrand factor, annexin, cadherin, selectin, and talin. Von Willebrand factor was one of the most abundant proteins in the coelomic fluid. In mammals, this protein binds to Factor VIII of the coagulation cascade and functions in platelet adhesion to collagen that becomes exposed at wound sites (Farndale et al., 2004). Annexin was also identified in abundance. This molecule functions in vertebrate blood to inhibit coagulation by competing with prothrombin or inhibiting thrombin. The abundance of these proteins in coelomic fluid suggests that the sea urchin wounding responses involve a vertebrate-like clotting reaction at the wound site that allows wound closure and sequestration of pathogens.

Among the other adhesion molecules identified, cadherins and selectins are calcium binding transmembrane proteins that play important roles in cell–cell adhesion (Frenette and Wagner, 1996). Cadherin (calcium dependent adhesion molecules) are frequent integral membrane molecules in mammalian tissues. Selectins, which are activated in inflammatory responses, have surface carbohydrate binding properties and mediate cell–cell adhesion in vertebrate blood cells. Talin in mammals functions to link integrins to the actin cytoskeleton (Becam et al., 2005), inhibits cadherin transcription (Becam et al., 2005), and is involved in phagocytosis (Allen and Aderem, 1996). The potential roles of cadherin, selectin and talin in sea urchin coelomic fluid is not clear, but they could be involved in the strong clotting reaction and coelomocyte phagocytosis that has been observed in this species.

One of the most abundant hypothetical proteins that we identified matched significantly to a set of galectins. Galectins are beta-galactoside binding lectins with homologous carbohydrate recognition domains (CRDs) that are potentialy involved in cell adhesion, migration, polarity, chemotaxis, proliferation, apoptosis, and differentiation (Barondes et al., 1994). Galectins have also been implicated in a number of host defense responses (Barondes et al., 1994). In bivalves, they are involved in parasite agglutination (Kim et al., 2008) and recognition (Tasumi and Vasta, 2007).

4.6. Stress response and detoxification

There were 27 matches to proteins involved in stress responses and detoxification. These proteins were relatively abundant (Fig. 3; Table 1; Supplementary data 3). Numerous heat shock proteins and chaperonin subunits were identified. Homologues of these proteins are found throughout the animal kingdom and their increased gene expression or protein concentration is often used as a marker for environmental stress (Fink, 1999). Heat shock proteins and chaperonins are involved in major growth-related processes such as cell division, transcription, translation, protein folding and transport, and they contribute directly to reducing cellular stress responses by preventing protein denaturation (Fink, 1999). Browne et al. (2007) have shown that extracellular HSP70 can suppress the immunological reactivity of sea urchin coelomocytes and induce the initiation of cleavage in fertilized eggs. This is in agreement with increasing evidence for the role of extracellular HSPs as modulators of immune functions (Schmitt et al., 2007).

Proteins similar to dual oxidase 1 and dual oxidase maturation factor also yielded high NSAFs values reflecting their abundance in the samples (Supplementary data 3). These proteins are involved in the production of hydrogen peroxide in vertebrate macrophages during host defense (Roos et al., 2003). The abundance of dual oxidase is increased in sea urchin coelomic fluid after LPS challenge (Dheilly et al., 2012) and it may promote the production of reactive oxygen species within the phagosomes of phagocytic coelomocytes, a primordial mechanism in antimicrobial defense.

Matches to proteins involved in iron metabolism were extremely abundant in the samples (Table 2; Supplementary data 3). For example, large quantities of transferrin, melanotransferrin, major yolk protein, ferritin and ceruloplasmin were identified. In many species, transferrin is primarily involved in iron metabolism and transport (Baker and Lindley, 1992). It is essential for promoting lymphocyte proliferation in many vertebrates (Brock and de Sousa, 1986), and is known to stimulate the nitric oxide responses in macrophages (Stafford and Belosevic, 2003). Major yolk protein and melanotransferrin, which are transferrin-like proteins, were the 8th and 9th most abundant proteins in coelomic fluid of the 307 proteins that were identified (Supplementary data 1). In mammals, melanotransferrin binds iron, which has led to speculation that it performs functions similar to transferrin (Baker and Lindley, 1992). However, recent data have shown that melanotransferrin expression is not directly related to iron uptake, and may instead be involved in protection against membrane lipid peroxidation (Sekyere and Richardson, 2000). Alternatively, melanotransferrin may have metalloprotease activity or act as an intercellular adhesion molecule by binding transferrin (Sekyere and Richardson, 2000). Studies on knockout mice showed that melanotransferrin has an important role in cell proliferation and migration that is unrelated to iron metabolism (Dunn et al., 2006). Another transferrin-like protein in sea urchins, major yolk protein (also called echinoferrin), shuttles iron in cells, but may also have other activities (Brooks and Wessel, 2002; Yokota et al., 2003).

Among the other proteins putatively involved in iron metabolism, ferritin has been characterised previously in the coelomic fluid of sea stars where it is thought to act as an acute phase protein during immune responses by sequestering iron to block bacterial proliferation (Beck et al., 2002). Ceruloplasmin also plays a role in iron metabolism in association with transferrin (Fox, 2003). The abundance of these iron-binding proteins and their functions in iron metabolism suggests that in sea urchins, as in vertebrates, iron plays a critical role in the immune system and defense against infection via cell proliferation, activation of the nitric oxide and reactive oxygen species responses or depletion of iron from body fluids. In vertebrates, iron sequestration is used to reduce iron availability to microorganisms within blood, tissues and phagosomes (Brock and Mulero, 2000; Ganz, 2009). The high abundance of iron sequestring proteins in coelomic fluid suggests a similar function in sea urchins.

4.7. Energy metabolism

There were 20 matches (3% of the coelomic fluid proteome) to proteins involved in energy metabolism. The most abundant of these were transaldolase and transketolase (Supplementary data 3), both of which function in the non-oxidative steps of the pentose phosphate pathway. In vertebrates, this pathway is the only mechanism for red blood cells to produce NADPH and to maintain the reduced state of glutathione, preventing cellular oxidation. However, in sea urchin coelomocytes, further studies are required to determine the potential role of the pentose phosphate pathway in the prevention of cellular oxidation during phagocytosis and/or in providing coelomocytes with the energy necessary to develop efficient immune responses.

4.8. Lysosome, proteases and peptidases

Seventeen proteins were identified as functioning within lysosomes, or acting as proteases and peptidases. Of these, the most abundant were a protease inhibitor, α2-macroglobulin, and a serine protease, thrombin. α2-Macroglobulin in vertebrates is involved in the immobilization and entrapment of proteases (Borth, 1992). These proteins were most likely soluble proteins present in the cell-free coelomic fluid. Vertebrate thrombin enzyme catalyses coagulation-related reactions by converting soluble fibrinogen into soluble strands of fibrin (Davie et al., 1991). Fibrin polymers then weave through loose platelet plugs crosslinking the clot. Together with α2-macroglobulin, calcineurin and amassins (described above), the presence of α2-macroglobulin and thrombin suggests that sea urchin coelomic fluid has a coagulation cascade with numerous similarities to that of vertebrates. Invertebrate forms of α2-macroglobulin also appear to have complement C3-like functions, such as opsonization, in addition to acting as protease inhibitors (Dodds and Alex Law, 1998).

4.9. Cell proliferation, reproduction and development

Our samples contained 17 matches to proteins that function in cell proliferation, reproduction and development, including numerous variants of echinonectin. Echinonectin is an adhesion protein that is present during embryonic development of echinoderms (Alliegro and Alliegro, 1991). It may also be involved in repair of the adult sea urchin skeleton (Mann et al., 2008). Echinonectin is known as a highly variable protein and there are 162 unique protein sequences similar to echinonectin in the NCBI database for *S. purpuratus* alone. Echinonectin consists of a series of coagulation factor 5/8 C terminal domains and discoidin-like C-type lectin domains (Alliegro and Alliegro, 2007). The significance of the high variability of echinonectin and of its domain structure in terms of embryonic development and immune responses remains unknown.

A significant concentration of apextrin was also identified in the coelomic fluid proteome. This protein has been identified previously in the secretory vesicles of sea urchin eggs and is potentially involved in cell adhesion (Haag et al., 1999). Interestingly, apextrin is overexpressed during acute immune responses against bacteria in both sea urchins and amphoxius (Huang et al., 2007; Dheilly et al., 2011b), but not in response to LPS challenge (Dheilly et al., 2012). Since apextrin is a member of the membrane attack complex and perforin superfamily, its expression profile may suggest a role in bacterial clearance.

4.10. Exchangers and ATPases

Thirteen ion exchange proteins and ATPases were identified (Supplementary data 3) of which a voltage dependent anion channel, a H^+ transporting ATPase, and a mitochondrial ATP synthase were the most abundant. Three homolgues of the mitochondrial solute carrier family members were also found. An EST encoding a vacuolar H^+ ATPase had been found previously in coelomocytes responding to LPS. Its presence was thought to be associated with the acidification of late endosomes and lysosomes during phagocytosis (Nair et al., 2005).

4.11. Intracellular transport

There were six matches to proteins involved in intracellular transport. These included major vault protein, adaptor protein complex 2, flotilin and sorting nexin (Supplementary data 3). Major vault protein is a large ribonucleoprotein involved in nucleocytoplasmic transport of ribosomes and/or mRNA (Hamill and Suprenant, 1997). It has been implicated in intracellular signaling and immune defense (Berger et al., 2009). During phagocytosis, major vault protien functions in vesicular transport and fusion of lysosomes and phagosomes. Similarly, the adaptor related protein 2 is part of the protein coat on the cytoplasmic side of vesicular membranes and links the budding vesicles to clathrin molecules that regulate endocytosis (Keyel et al., 2008). Flotilin is involved in vesicular trafficking and colocalizes with cathepsin, suggesting it accumulates in lysosomes (Jeong et al., 2007). The function of sorting nexin is unclear, but it may also be involved in vesicular trafficking during phagocytosis (Leprince et al., 2003). Vesicular and membrane trafficking are important processes involved in secretion and in cell restructuring and the formation of filopodia, endocytosis and lysosome fusion. The importance of these functions in sea urchin coelomocytes may be reflected by the relative abundance of proteins in this category.

5. Conclusions

The data that we present here provides an initial overview of the proteins expressed within the coelomic fluid of purple sea urchins. It paves the way for controlled experiments to study the proteins that are specifically affected over the course of immune responses in different coelomocyte types and in cell-free coelomic fluid. In order to increase coverage and identify proteins expressed at lower levels, future studies should focus on the proteome of different types of coelomocytes, and eventually, on different subcellular fractions (i.e., cytoplasmic, micochondrial, nuclear, membrane associated, etc.). Because sea urchins show significant genetic variability (Sodergren et al., 2006), previous analyses on *S. purpuratus* immunity at the transcriptomic level were undertaken on a individual sea urchins before and after challenge (Nair et al., 2005; Terwilliger et al., 2007) to avoid inherent problems associated with inter-individual diversity in the immune response. To pave the way for a more thorough proteomic analysis, in this study we compared the data obtained from three sea urchins. The reproductibility of the data was tested by plotting the log (NSAF) values of the three individuals against each other. The three linear curves yielded a high r^2 values demonstrating that the proteomes of the three animals used in this study were similar.

Previous transcriptomic analysis of the immune response of sea urchins to challenge with LPS were carried out by end sequencing clones to generate EST databases (Smith et al., 1996; Nair et al., 2005). A significant number of clones encoding immune related proteins were identified among the ESTs, as well as a large array of clones encoding proteins involved in cytoskeletal modulation. One surprising result was the abundance of ESTs that matched to *Sp185/333* sequences (73%). In the present study, the most striking difference compared to the previous EST analyses was the low number of peptides identified that matched to Sp185/333 sequences. The low abundance of Sp185/333 proteins suggests that only a fraction of *Sp185/333* transcripts may be translated into proteins. Alternatively, the high intra- and inter-individual variability of these molecules may have been such that the Sp185/333 proteins expressed by the three individuals in our study had not been previously sequenced and consequently were not in the database used to perform Blast searches and so were not identified. Difficulties in identifying Sp185/333 proteins by MS has been discussed previously by Dheilly et al. (2009).

Other gene families that are also highly diversified in sea urchins, such as TLRs, NLRs and fibrinogen domain containing proteins, were not found in our samples. This suggests that these genes are expressed at very low levels in sea urchin coelomic fluid under the experimental conditions that we tested. Alternatively, the relatively low identification of highly variable proteins such as Sp185/333 proteins, TLRs, NLRs and fibrinogen domain containing proteins may be a limitation of the technique to identify variants express at low levels. Indeed, an overall high quantity of each of these families may be expressed but the abundance of each individual variant may be below detectable levels. In contrast, the current study identified numerous SRCRs, suggesting either that this family of highly variable proteins plays a major role in the function of sea urchin coelomocytes or that SRCRs are less diversified than the other families discussed above.

One can argue that receptors are transmembrane proteins and as such may be more difficult to extract than soluble cytoplasmic proteins. Cell subfractionation may be performed to investigate the coelomocyte membrane proteome and to enrich the samples for pathogen recognition receptors. Alternatively, analyses of such highly variable immune response molecules could be performed at the transcriptomic level using new sequencing technologies (Morozova et al., 2009). De novo assemblers are sensitive enough to recover spliced isoforms and transcripts from recently duplicated genes (Grabherr et al., 2011; Schulz et al., 2012) and may be able to overcome the difficulty inherent from the high intra- and inter-individual variability of highly variable protein families.

In addition to SRCRs, a number of other interesting molecular pathways involved in intracellular processes linked to immune functions included components of a complement system, lipid metabolism, and a putative clotting pathway. It remains to be seen what role these systems play in the immune responses of sea urchin, and how they interact with each other. Further studies have been undertaken to identify the proteins expressed in *H. erythrogramma* coelomic fluid overtime after infection (Dheilly et al., 2012), and other experiments are under way to identify the specific proteomes of the different, functionally specialized types of coelomocytes. When combined with the current data, these studies will provide a better picture of the coordinated and integrated functions of the different types of coelomocytes found in coelomic fluid.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2013.01.007.

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