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Article *in* Developmental and comparative immunology · August 2010

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SpTie1/2 is expressed in coelomocytes, axial organ and embryos of the sea urchin *Strongylocentrotus purpuratus*, and is an orthologue of vertebrate Tie1 and Tie2

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ARTICLE INFO

Article history:
Received 29 December 2009
Received in revised form 25 March 2010
Accepted 26 March 2010
Available online 10 April 2010

Keywords:
Echinoderm
Receptor tyrosine kinase
TEK
Coelomocyte
Axial organ
Embryo

ABSTRACT

A full length cDNA sequence expressed in coelomocytes shows significant sequence match to vertebrate Tie1 and Tie2/TEK. Vertebrate Tie2/TEK is the receptor for the angiopoietins and plays an important role in angiogenesis and hematopoiesis, whereas Tie1 regulates the activity of Tie2. The deduced sequence of the SpTie1/2 protein has a similar order and organization of domains to the homologous vertebrate proteins including a highly conserved receptor tyrosine kinase domain in the cytoplasmic tail. The N terminus of the ectodomain has one immunoglobulin (Ig)-Tie2_1 domain, followed by an Ig domain, four epidermal growth factor domains, a second Ig domain, and three fibronectin type III domains. The *SpTie1/2* gene is expressed in coelomocytes and the axial organ, whereas other organs do not show significant expression. The timing of embryonic expression corresponds with the differentiation of blastocoelar cells, the embryonic and larval immune cells. Searches of the sea urchin genome show several gene models encoding putative ligands and signaling proteins that might interact with SpTie1/2. We speculate that SpTie1/2 may be involved in the proliferation of sea urchin immune cells in both adults and embryos.

1. Introduction

Echinoderms are frequently used as the evolutionary outgroup in studies comparing deuterostome relationships and the evolution of vertebrate characteristics. Sea urchins, as members of the echinoderm phylum, are easily obtained, housed, handled, spawned and otherwise manipulated, and are frequently used in investigations of early development (Davidson, 2006; Sodergren et al., 2006a,b). The echinoderms, as the sister phylum to the chordates, are an important basal group for making evolutionary inferences about the immune system in deuterostomes (reviewed in Rast and Messier-Solek, 2008). Adult echinoderms differ significantly in their structure from chordates, in that they possess a unique water vascular system and rely on a large open coelomic cavity rather than a closed circulatory system with a heart and blood vessels (Hyman, 1955). Yet, there are aspects of their immune system that show similarities to innate immunity in vertebrates including complement homologues, Toll-like receptors, NACHT/NOD-like receptors, and a variety of lectins (Smith et al., 1999, 2001, 2006; Hibino et al., 2006; Rast et al., 2006). The cells in the coelomic cavity, the coelomocytes, are a mixture of different morphologically identifiable types of which the majority resemble phagocytic cells of vertebrates in their participation in innate immune functions such as phagocytosis, encapsulation, and cellular clotting in response to injury (Smith et al., 2006; Johnson, 1969; Smith and Davidson, 1994; Gross et al., 1999). By investigating conserved aspects of immune function in the immune cells of both vertebrates and echinoderms, we may be able infer the origins and evolution of the basic, underlying immunity that is present in animals within the deuterostome lineage.

Abbreviations: Ig, immunoglobulin; RTK, receptor tyrosine kinase; Tie, tyrosine kinase with Ig and EGF domains; TEK, tunica interna endothelial cell kinase; Ang, angiopoietin; EST, expressed sequence tag; FGFR, fibroblast growth factor receptor; GFR, growth factor receptor; SMART, simple modular architecture research tool; PI3K, phosphoinositide 3-kinase; LPS, lipopolysaccharide; wCF, whole coelomic fluid; CMFSW-EI, calcium- and magnesium-free sea water with EDTA and imidazole; Ct, cycle threshold; MOPS, 3-(N-morpholino)propanesulfonic acid; SDS, sodium dodecylsulfate; EDTA, ethylenediaminetetraacetic acid; SSC, sodium chloride and sodium citrate; BSA, bovine serum albumin; nt, nucleotide(s); UTR, untranslated region; EGF, epithelial growth factor; FNIII, fibronectin type III; qPCR, quantitative PCR; hpf, hours post-fertilization; HSCs, hematopoietic stem cells; LTR-HSCs, long-term repopulating HSCs; MAPK, mitogen-activated protein kinase; WGS, whole genome shotgun; BCM, Baylor College of Medicine; SNPs, single nucleotide polymorphisms.

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doi:10.1016/j.dci.2010.03.010

A hallmark of the vertebrate immune system is cellular interactions mediated through ligand interactions with receptors displayed on the cell surface. One of the major families of receptors involved in regulation of cellular activity and proliferation in chordates is that of the receptor tyrosine kinases (RTKs) (Schlessinger, 2000; Fantl et al., 1993; Simon, 2000). Upon binding ligands, these receptors dimerize and induce cross-phosphorylation of specific tyrosine residues in the cytoplasmic tail. The phosphotyrosines initiate a signaling cascade that causes a set of responses specific to the cell and to the receptor, with the main targets being regulation of transcription and modulation of the cytoskeleton. A recent inventory of RTKs encoded in the genome of the sea urchin, *Strongylocentrotus purpuratus*, has shown that most families of vertebrate RTKs are also represented in sea urchins (Sodergren et al., 2006b). Although many RTK families in vertebrate genomes have multiple members, sea urchins typically have a single orthologue for all but two of these families (Lapraz et al., 2006). Similar results have been reported for amphioxus (D'Aniello et al., 2008).

A specific RTK family, consisting of two vertebrate genes, *Tie*1 and *Tie*2/*TEK*, encode proteins with a unique arrangement of extracellular domains, and function in hematopoietic and blood vessel forming tissues (Dumont et al., 1992; Maisonpierre et al., 1993; Partanen et al., 1992; Sato et al., 1993; Martin et al., 2008). These two receptors seem to be present universally in vertebrates, where they are expressed in the cell lineage that forms the endothelium of blood vessels (Dumont et al., 1994a,b; Sato et al., 1995, reviewed by Jones et al., 2001) and develops into the stem cells for hematopoiesis (Hsu et al., 2000; Tachibana et al., 2005; Takakura et al., 1998; Batard et al., 1996; Hashiyama et al., 1996). *Tie*1 and *Tie*2 are expressed in endothelial cells (Schnurch and Risau, 1993), particularly in normal cells undergoing neovascularization (Korhonen et al., 1995) and in metastatic melanoma endothelial cells (Kaipainen et al., 1994). The Tie receptors also are expressed in early stages of hematopoiesis (Batard et al., 1996; Iwama et al., 1993) and appear to be necessary for proliferation of blood cells in adult bone marrow, but do not function in embryonic or fetal hematopoietic tissues (Puri and Bernstein, 2003). The angiopoietin (Ang) family of ligands, including Ang1 (Davis et al., 1996) and Ang4 (Jones et al., 2001) interact with Tie2 and activate tyrosine phosphorylation activity. Ang2 is usually a negative regulator of the tyrosine kinase function (Jones et al., 2001; Huang et al., 1999), but sometimes enhances the downstream signaling activity of Ang1 (Huang et al., 1999). No ligand has been positively identified for Tie1, but Ang1 and Ang3/4 promote its phosphorylation in cells co-transfected with both *Tie*1 and *Tie*2 genes (Yuan et al., 2007). The critical role of Ang1 and Tie2 in non-fetal hematopoiesis appears to be maintenance of a quiescent, anti-apoptotic population of hematopoietic stem cells that are protected from stresses associated with active proliferation and adhere to osteoblasts in the bone marrow (Arai et al., 2004).

During an initial expressed sequence tag (EST) study of coelomocytes from the purple sea urchin, *S. purpuratus*, EST059 matched to the cytoplasmic domain of an RTK (accession number R61943)(Smith et al., 1996). BLAST matches suggested that the sequence fragment encoded a growth factor receptor or Tie receptor. The full length cDNA sequence, *Sp059*, corresponded to a single gene model identified from the *S. purpuratus* genome that encodes a member of the Tie family designated *SpTie1/2* (Sodergren et al., 2006b; Hibino et al., 2006; Bradham et al., 2006). The combination of the suite of extracellular domains, the highly conserved cytoplasmic domain, plus a phylogenetic analysis of the tyrosine kinase domain from Tie1, Tie2 and other RTK growth factor sequences suggested that sea urchin SpTie1/2 is an ancestral Tie1 and Tie2 homologue, in agreement with gene annotations (Sodergren et al., 2006b; Hibino et al., 2006; Lapraz et al., 2006; Bradham et al., 2006). The *SpTie1/2* gene is highly expressed in coelomocytes, axial organ, and embryos, suggesting an involvement in immune cell proliferation in adults, in addition to a time in development when embryonic secondary mesenchyme cells differentiate into blastocoelar cells, which are the immune cells of embryos and larvae (Smith et al., 2006; Tamboline and Burke, 1992; Silva, 2000; Furukawa et al., 2009).

2. Methods

2.1. Care and treatment of sea urchins and embryo cultures

Sea urchins, *S. purpuratus*, were supplied by Marinus Scientific Inc. (Long Beach, CA), the Southern California Sea Urchin Company (Corona Del Mar, CA) or Westwind Sealab Supplies (Victoria, British Columbia, Canada) and maintained as previously described in a closed aquarium at 14 °C with filtered sea water sterilized with UV light (Gross et al., 2000; Shah et al., 2003). Immune activation was induced in adult sea urchins by injections of lipopolysaccharide (LPS) as described (Terwilliger et al., 2007; Smith et al., 1995). Gametes were collected after KCl injection to induce spawning and fertilized embryos were cultured in 0.45 μM filtered Instant Ocean in suspension flasks at 15 °C.

2.2. Immunoquiescent and immunologically activated sea urchins

Immunoquiescent sea urchins were maintained without manipulation according to Nair et al. (2005). Immunoquiescent animals were injected with LPS (2 μg/ml of coelomic fluid) (Sigma–Aldrich, St. Louis, MO) as described (Terwilliger et al., 2007; Smith et al., 1995) to generate immunologically activated animals. Whole coelomic fluid (wCF) samples were collected before challenge with LPS and 24 h after challenge, and tissues were collected from animals that had not been challenged or were collected 15 days after challenge. Animal #8 was considered non-immunoquiescent because samples were taken within 24 h of shipment.

2.3. cDNA libraries and screening

Clones for sequencing were obtained by screening two coelomocyte cDNA libraries. The first was a conventional, random primed, directionally cloned λExCell cDNA library that was constructed from mRNA collected from 18 sea urchins, and excised and stored as a plasmid library (Smith et al., 1996; Al-Sharif et al., 1998). The second was an arrayed cDNA library constructed from mRNA collected from non-activated coelomocytes from six sea urchins and cloned into pSPORT (Cameron et al., 2000; Rast et al., 2000). The libraries were screened with riboprobes (see below) as described (Al-Sharif et al., 1998; Multerer and Smith, 2004).

2.4. Cycle sequencing

Cycle sequencing was performed on the pSPORT clones using the Big Dye kit (Perkin-Elmer/Applied Biosystems, Foster City, CA) with Sp6 and T7 primers plus internal primers (Operon Technologies, Huntsville, AL) (Table 1) and analyzed on a single capillary 373A automated sequencer (Applied Biosystems, Foster City, CA) or by the Nucleic Acid Core Facility of the Medical College of Wisconsin. The overlapping sequences were aligned using the DNAsis sequence analysis program (Hitachi, Yokohama, Japan). The consensus sequence was generated by comparing the cDNA sequences and whole genome shotgun (WGS) trace sequences from the sea urchin genome available at the NCBI website (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=7668). The final *Sp059* cDNA sequence was submitted to GenBank with accession number GQ979611.

Table 1
Primers used in qPCR.

Primer	Sequence	Target cDNA
SpTie1/2F	CGTCAAGACGCTGAAAGATG	Coelomocyte
SpTie1/2R	CACAATGTTGGGGTGTGTTC	Coelomocyte
Tie1-2 QPCRF	CATCCCTAACCCTGAAGGTG	Embryo
Tie1-2 QPCRR	TGGAATTTCGCTCTCTGGTC	Embryo
SpL8rev	CACAACAAGCACAGGAAGGGA	Coelomocyte
SpL8for	AGCGTAGTCGATGGATCGGAGT	Coelomocyte
18S-F	CAGGGTTCGATTCCGTAGAG	Embryo
18S-R	CCTCCAGTGGATCCTCGTTA	Embryo
Sp6	GATTTAGGTGACACTATAG	Sequencing
T7	TAATACGACTCACTATAGGG	Sequencing

2.5. Assembly of the gene sequence from gene models; determination of structural domains for the encoded protein

The genomic scaffolds containing the *SpTie1/2* gene were identified by BLAST searches against the sea urchin genome (v2.1) at NCBI, and the Baylor College of Medicine (BCM) Spurpuratus BAC plus WGS Assembly (v2.0, June 15, 2006 (http://www.hgsc.bcm.tmc.edu/blast/blast.cgi?organism=Spurpuratus). Exons were identified by aligning the cDNA to the genomic sequence, and verified by the presence of GT-AG splice signals located at the ends of the introns. The *Sp059* cDNA sequence was used to search GenBank by BLASTx to identify homologues from other species. Structural domains in the deduced protein were determined with the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/) in the normal mode.

2.6. Sequence alignments and phylogenetic analyses

The SpTie1/2 protein sequence was used in tBLASTx searches of GenBank to identify the most similar sequences in vertebrates and invertebrates for phylogenetic analysis. The tyrosine kinase domain was employed in an alignment in ClustalW using the default parameters (Thompson et al., 1994) with subsequent manual editing in BioEdit (Hall, 1999). A minimum evolution tree with 1000 bootstrap iterations was constructed in MEGA4 (Kumar et al., 2004). Other phylogenetic methods were used to confirm the results (see the legend of Fig. 8).

The sea urchin genome (v2.0) was searched for the Ang homologue by BLASTp using four vertebrate angiopoietin sequences; human Ang1 (accession number NP_001137.2), human Ang4 (NP_001138), *Danio rerio* Ang1 (NP_571888), and *Danio rerio* Ang2 (NP_5711889.1). Sea urchin gene models that were present in each of the four BLASTp analyses with scores of e^{-06} or better were selected for further analysis. Gene models encoding proteins with domains other than a signal sequence and one fibrinogen domain were eliminated. The fibrinogen domains from the putative sea urchin Ang sequences ($n = 30$) were identified using SMART and aligned to the vertebrate Ang domains ($n = 14$) with ClustalW (Thompson et al., 1994) and used for phylogenetic analyses (MEGA 3.1; Kumar et al., 2004) with 1000 bootstraps. Similar analyses were done with the Ig_Tie2_1 domain from the Tie2/TEK sequences and the corresponding region from the Tie1 sequences, in addition to the first Ig domain from both the Tie1 and Tie2/TEK sequences.

Sea urchin gene models encoding Grb homologues were identified using BLASTp with the human Grb sequence (CAG29359) by searching the *S. purpuratus* genome (v2.1). The sea urchin p85 gene model (XP_793526) was identified by BLASTp searches of the *S. purpuratus* genome (v2.1) using the human p85 subunit of phosphoinositide 3-kinase (PI3K) (AK302049).

2.7. Total RNA isolation

wCF samples were withdrawn from adult sea urchins and diluted (1:3) into calcium–magnesium-free sea water with 70 mM EDTA and 50 mM imidazole pH 7.4 (CMFSW-EI) (Terwilliger et al., 2006). Each sample, with 10^5 to 10^7 coelomocytes per ml, was centrifuged at $10{,}000 \times g$ for 1–2 min at 4 °C and the cell pellet was overlain with RNAlater® (Ambion Diagnostics, Austin, TX). Tissue samples (pharynx, gut, gonad, esophagus, and axial organ) were obtained by dissection and transferred immediately to RNAlater®. Total RNA was extracted from coelomocytes and tissue samples using the RNeasy® Micro or Mini Kit (Qiagen, Valencia, CA) according the manufacturer's directions, followed by treatment with RQ1 DNAse (Promega, Madison, WI) to digest any contaminating genomic DNA. RNA (0.75–1 μg) was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's directions.

Embryos were collected at the several different time points and total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA), treated with DNase using the DNA-free kit (Ambion Diagnostics, Austin, TX). RNA (2 μg) was reverse transcribed into cDNA using TaqMan® Reverse Transcription Reagents (Applied Biosystems) in 100 μl reactions.

2.8. Quantitative PCR

SpTie specific primers (Table 1) were used to amplify a region of the *SpTie1/2* cDNA in coelomocytes and embryos. SpL8for and SpL8rev primers (Table 1) were used to amplify a region of the *SpL8* cDNA in coelomocytes. The *SpL8* (EST219; R62029) is a homologue of the human *L8* gene that encodes a ribosomal protein (Smith et al., 1996). 18S-F and 18S-R primers (Table 1) were used to amplify the 18S ribosomal cDNA in embryos. Primers for qPCR were designed using Primer Premier software (Premier Biosoft International, Palo Alto, CA). Approximately equivalent volumes of the reverse transcriptase (RT) reactions were amplified with both SpTie1/2 primers and SpL8 primers using the same annealing temperature (60 °C). The standard curve for *SpL8* was generated with five 10-fold serial dilutions (10^3 to 10^7 copies per reaction) of a cDNA clone of *SpL8*. The *SpTie1/2* standard curve was generated with five 10-fold serial dilutions (10^2 to 10^6 copies per reaction) of a control *Sp059* cDNA clone (pSPORT-179C22; 80 nt amplicon from position 3460–3540 in the tyrosine kinase region [see Fig. S1]). The 18S primers were used on embryos to evaluate the total cDNA input for each reaction according to standard protocols. Each qPCR reaction (25 μl) consisted of 1× Absolute QPCR SYBR Fluorescein Mix (ABgene, Surrey, UK), 200 nM of each primer, and 0.025–0.1 μl of the RT reaction. cDNA samples from adults were amplified in an iCycler (Bio-Rad Laboratories, Hercules, CA) using the following conditions: 12 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 59 °C for 45 s, followed by a hold at 4 °C. An ABI Prism 7000 Sequence Detection System was used to measure the embryonic *SpTie1/2* message prevalence with the following amplification parameters: 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Dissociation curves were consistent with a single amplicon for all samples. Cycle threshold (Ct) values for cell and tissue cDNAs were plotted on the standard curves to determine the starting quantity of messages in each sample. *SpL8* is constitutively expressed in all tissues (Multerer and Smith, 2004), as is the 18S ribosomal RNA gene, and these cDNAs were used to normalize variations in cDNA concentration. Relative *SpTie1/2* gene expression was determined by calculating the ratio of *SpTie1/2* starting quantity to *SpL8* or 18S starting quantity.

Data obtained by qPCR from adult cDNA samples were analyzed statistically using SAS ver 9.1.3 software (SAS Inc., Carey, NC). Statistical significance was assessed by analysis of variance (Lebedev et

al., 2005) in the linear general models regression procedures. Bonferroni correction was used in the pairwise means comparison to contrast groups of values, such as different treatments, organs, and individual animals. Experimental error was addressed by including duplicate measurements among model parameters.

2.9. Genome blots and riboprobe

Genomic DNA was isolated from sperm from three sea urchins according to (Lee et al., 1984), digested to completion with EcoRI, HindIII or PstI and separated on a 0.75% agarose gel. The gel was blotted by capillary action onto GeneScreen Plus (New England Nuclear) according to standard protocols (Sambrook et al., 1989). Filters were baked and prehybridized (5× SSC, 10 mM EDTA, 5× Denhardt's solution [5% ficoll, 5% BSA, 5% SDS, 5% polyvinyl pyrrolidone], 50% formamide) prior to incubation with the riboprobe.

The template for the riboprobe was a 850 bp SmaI/HindIII fragment of pSPORT-81M18 that was subcloned into pBluescript (Stratagene, La Jolla, CA) and spanned from the middle of the Ig-Tie2_1-like domain through the second EGF domain. The pBsc-M18a subclone was linearized with SmaI and 555 ng was used as the template to produce a ^{32}P-labeled riboprobe to screen the Southern blot according to (Multerer and Smith, 2004; Terwilliger et al., 2004). The probe was heated to 70 °C for 4 min prior to being added to the filters for hybridization overnight at 42 °C in a rotating oven (Robbins Scientific). Final washes of the filters were conducted at 65 °C in 1× SSC with 1% SDS, followed by exposure to X-OMAT AR film (Eastman Kodak Co., Rochester, NY) without an intensifying screen. Films were scanned with a Astra 4000 flatbed scanner (Umax Technologies, Inc., Taiwan) and the digital images were formatted in Photoshop (Adobe Systems Inc., San Jose, CA).

3. Results

3.1. The full length Sp059 cDNA sequence

The first EST project conducted on purple sea urchin coelomocytes identified EST059, which spanned the 3′ end of a mRNA encoding the tyrosine kinase domain of an RTK (Smith et al., 1996). The EST was reported as similar to epidermal growth factor receptor or platelet derived growth factor receptor, although it was also similar to Tie receptors. Two coelomocyte cDNA libraries were screened yielding 38 positive clones of which six clones were chosen for sequencing based on clone insert sizes and restriction mapping, and assembled into a cDNA with a single open reading frame called *Sp059* (GenBank accession number GQ979611). The cDNA sequence was 4713 nucleotides (nt) in length, including a 5′ untranslated region (UTR; 387 nt), a coding region of 4296 nt, and a short 3′ UTR (29 nt) (Fig. S1). The 3′ UTR appeared to be incomplete because a poly A stretch was not identified, likely a result of library construction using a random primer, which typically does not capture the entire 3′ end of messages. Subsequent searches of the sea urchin genome identified the predicted full length 3′ UTR sequence based on a polyadenylation site located 1273 nt 3′ of the stop codon. A genome search was also used to identify additional 5′ UTR sequence of 725 nt between the first start codon and the putative first rA that might be incorporated into the transcript and located 25 nt 3′ of the TATAA box. With the cDNA and genomic sequence added together, the full length mRNA was estimated to be 6294 nt. Three start codons were present in the 5′ UTR, which were followed by in-frame stop codons. Within the coding region of *Sp059*, there were two possible start codons separated by 6 nt, however, the second was imbedded within a Kozak sequence (Kozak, 1987), and therefore may be more commonly used for initiating translation. Thus, *Sp059* may encode a protein of 1433 or 1436 amino acids, depending on which start codon is used.

The libraries from which the *Sp059* cDNA clones were isolated were constructed from coelomocyte RNA pooled from 18 sea urchins (Smith et al., 1996; Al-Sharif et al., 1998) and a second that was constructed from six sea urchins (Cameron et al., 2000; Rast et al., 2000). Consequently, as many as 48 alleles may have been used to assemble the cDNA sequence plus the two alleles from the genome. More than half of the single nucleotide polymorphisms (SNPs) (125 of 239 nt) in the *Sp059* sequence encoded nonsynonymous changes, including 67 that changed the type of amino acid (Fig. S1). This represented a polymorphism frequency of 5.5% in the cDNA sequence and 2.9% in the protein sequence.

3.2. Gene copy number and the SpTie1/2 gene model

Several vertebrate genomes have been investigated for the presence of Tie homologues, and two genes are consistently present; Tie1 and Tie2/TEK (Jones et al., 2001). However, during the process of annotating the sea urchin genome, one gene model, SPU_024044 was reported as encoding *SpTie1/2* (Sodergren et al., 2006b; Hibino et al., 2006). This sequence was missing some of the exons that matched to the cDNA sequence, while a second gene model SPU_026748, was missing other exons. The two gene models were used to generate a corrected SPU_024044 gene model that included all exons encoding sequences in the cDNA. The two gene models were likely the result of miss-assembly of a single gene. Based on comparisons to the cDNA sequence, *SpTie1/2* gene model had 27 exons, which was significantly more than the average sea urchin gene with 8.3 exons (Sodergren et al., 2006b). Sizes and characteristics of the introns differed among genome builds and therefore are not described. In agreement with the single gene model, a genome blot of three individual sea urchins showed two or three bands for each digest, which was consistent with a single copy gene for *SpTie1/2* (Fig. 1).

3.3. The deduced SpTie1/2 protein

The deduced SpTie1/2 amino acid sequence was used to search the non-redundant sequences in GenBank using BLAST, and the best match was to the Tie proteins in vertebrates (Jones et al., 2001; Peters et al., 2004). Domain analysis of the deduced protein using SMART showed that there were two hydrophobic regions in SpTie1/2; a N terminal signal sequence of 19 amino acids and a transmembrane region of 21 amino acids (Figs. S1, 2). The extracellular region of the protein had three types of domains, immunoglobulin (Ig), epithelial growth factor (EGF) and fibronectin

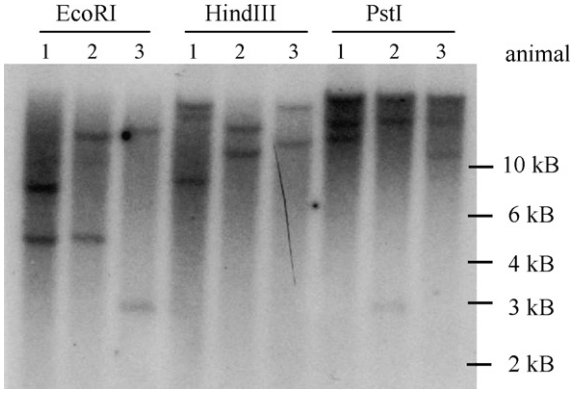

Fig. 1. *SpTie1/2* is a single copy gene. Genomic DNA from sperm collected from three sea urchins was digested to completion with three restriction enzymes, electrophoresed and blotted. The filter was analyzed with a riboprobe generated from a cDNA template pBsc-M18a (see legend of Fig. 2 for details). Each lane shows two or three bands, consistent with a single copy, heterozygous locus. Standards are indicated to the right.

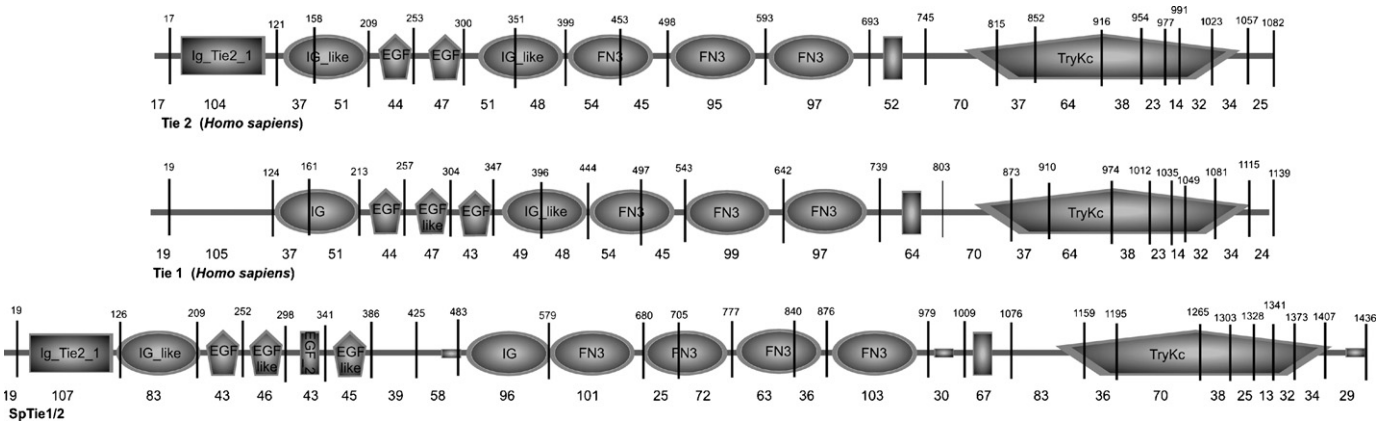

Fig. 2. Functional domains and exon positions in SpTie1/2 compared to human Tie1 and Tie2. Vertical lines indicate locations of introns. Numbers above each figure indicate the C terminal amino acid for each exon. Numbers below each figure indicate the number of amino acids encoded by each exon. Three different types of immunoglobulin domains are indicated by Ig_Tie2_1, IG_like and IG. The wide vertical bar represents the transmembrane domain. EGF and EGF-like, epithelial growth factor domains; FNIII, fibronectin 3 domains; TryKc, tyrosine kinase domain. Modified from figures generated by Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/). HsTie1 GenBank accession number is P35590; HsTie2 GenBank accession number is BAG58094.

type III (FNIII) domains, that were in the same order as those in vertebrate Tie1 and Tie2/TEK proteins (Sato et al., 1993). The first two Ig domains were separated from the third by three EGF domains and one EGF2-like domain, which were followed by four FNIII domains (Fig. 2). SpTie1/2 was similar to vertebrate Tie2 in having three Ig, but differed in having four EGF and four FNIII domains, while the vertebrate proteins have only two or three EGF domains and three FNIII domains. The second and third Ig domains in SpTie1/2 had conserved N-linked glycosylation sites at amino acid 167 (NATR) and 519 (NVTL), with another conserved site located between domains at amino acid 481 (NVTG) (Fig. S1). The tyrosine kinase domain in the cytoplasmic tail contained a typical catalytic site, ATP-binding site, and activation loop showing a high degree of sequence similarity between the sea urchin and vertebrate sequences (Fig. 3). Overall, the types, numbers and relative organization of the encoded domains in addition to BLAST results indicated that the sea urchin sequence was a Tie homologue.

3.4. Exons in SpTie1/2 correlate with the protein domains

The *SpTie1/2* gene model was composed of 27 exons and a mean length was 158 nt, a range of 37–306 nt, and a median length of 132 nt. Many of the exons in the *SpTie1/2* gene model encoded separate domains in the protein (Figs. S1, 2). The signal sequence (exon 1), the three Ig domains (exons 2, 3, 10), the EGF domains (exons 4, 5, 6, 7), the first and fourth FNIII domains (exons 11, 16), the transmembrane region (exon 18), and the C terminal cytoplasmic tail (exon 27) were all encoded by single exons. The remaining

Fig. 3. Alignment of the receptor tyrosine kinase domains and cytoplasmic tails of SpTie1/2, Tie1 and Tie2. The Tie1 and Tie2 sequences are mammalian consensus sequences. Capital letters designate highly conserved positions in which the same amino acid is shared among all mammalian Tie1 or Tie2 proteins. Bold script indicates positions that are conserved in both vertebrate consensus sequences and in SpTie1/2. Underlining indicates the major functional domains generally present in RTKs as determined from the NCBI Structure database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi). Exon boundaries are indicated by gaps, and the phase of intron insertion is designated by a number. The exon numbers refer to the *SpTie1/2* exons. Asterisks denote positions of tyrosines in the cytoplasmic tail of vertebrate Tie2 proteins.

domains in SpTie1/2 were encoded by two or more exons, including the second and third FNIII domains (exons 12 and 13, 14 and 15) and the tyrosine kinase domain (exons 19-26; Figs. S1, 3). Exons 8, 9, and 17 did not encode discernable domains for a total of 27 exons in *SpTie1/2* compared to 22 exons present in human *Tie* genes (Fig. 2).

The tyrosine kinase domain in the cytoplasmic region was the most highly conserved region of the Tie sequences. The domain was encoded by eight exons for the sea urchin, which is the same for the vertebrate genes (Figs. 2 and 3). As in the human *Tie* genes, the functional regions of the tyrosine kinase domain were encoded by separate exons; the ATP-binding site in exon 21, the catalytic site in exon 22, and the activation loop in exons 22 and 23 (Fig. 3). Positions of all seven introns within the tyrosine kinase domain of *SpTie1/2* were identical to those in the human *Tie* genes, as was the length of all exons except exon 21 for *SpTie1/2*, which had an insertion that encoded an additional six amino acids. The intron position pattern for the *Tie* genes is unique and different from other RTK families, which have different numbers of exons and at least one intron position that is different (e.g., D'Aniello et al., 2008) (Fig. 3). Consequently, conservation of the positions of the introns within *SpTie1/2* and the vertebrate genes indicates homology. A similar result was found for the tyrosine kinase domains in the *BfTie* homologues in *Branchiostoma floridae* (D'Aniello et al., 2008), however the types, numbers and organization of the ectodomains for these two proteins were not well conserved.

When the boundaries of the other SpTie1/2 domains and exons were compared to the human Tie sequences, a number of additional similarities and some differences were noted (Fig. 2). Each of the Ig domains in the vertebrate Tie proteins was encoded by two exons, while the Ig domains in SpTie1/2 were encoded by single exons. There were two or three EGF domains in the human Tie sequences, whereas SpTie1/2 had four. SpTie1/2 had four FNIII domains, while both human sequences had three, and each was encoded by one or two exons. Phylogenetic analysis of the FNIII domains from human Tie1, Tie2 and SpTie1/2 showed that the first two FNIII domains from SpTie1/2 consistently clustered together, suggesting that they were either the result of exon duplication and were very similar, or were both very different from the third and fourth FNIII domains in SpTie1/2 and the vertebrate sequences (results not shown). Overall, comparison of exons encoding domains suggested that the sea urchin *Tie* gene has undergone some diversification in the ectodomain. However, the conservation of the exons and the intron positions within the tyrosine kinase domain lent strong inference to the notion that *SpTie1/2* is a *Tie* homologue.

3.5. SpTie1/2 is expressed in coelomocytes, axial organ and embryos

To understand the gene expression patterns of *SpTie1/2*, quantitative PCR (qPCR) was used to analyze the *Sp059* mRNA content in coelomocytes, as well as tissue samples from axial organ, gut, gonad, pharynx, and esophagus. Relative *SpTie1/2* gene expression was determined by calculating the ratio of *Sp059* starting quantity with respect to *SpL8* (ribosomal L8 homologue) starting quantity. Results indicated that the highest *SpTie1/2* gene expression was in the axial organ, followed by the coelomocytes, and was lowest in the other organs ($P < 0.05$; Fig. 4). The expression in axial organ was significantly higher than in coelomocytes ($P < 0.05$) and the increase in axial organ was correlated with immune challenge ($P < 0.05$). No association was observed between *SpTie1/2* gene expression and immune challenge in coelomocytes or in the other organs. These results confirmed previous results for *SpTie1/2* gene expression in coelomocytes (Smith et al., 1996) and demonstrated that it was also expressed in the axial organ.

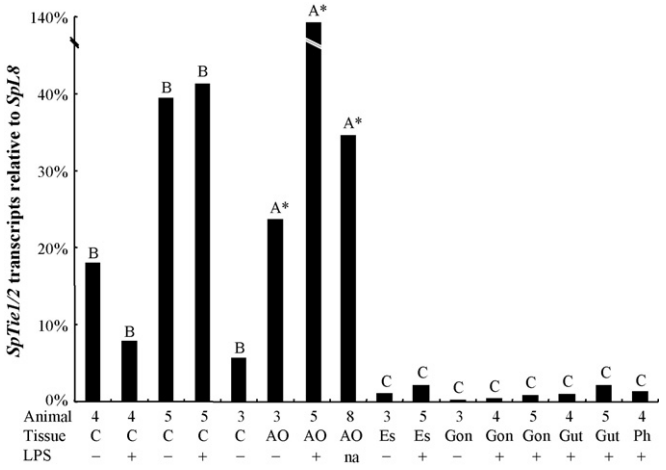

Fig. 4. *SpTie1/2* expression in adult sea urchins is specific to coelomocytes and axial organ. Coelomocytes and various tissues were collected from adult sea urchins (3, 4, 5 and 8) and analyzed for *SpTie1/2* gene expression by qPCR. Total RNA was extracted from axial organ (AO), esophagus (Es), gonad (Gon), gut (Gut), pharynx (Ph) and coelomocytes (C). Coelomocyte samples from animals 4 and 5 were collected before (–) and 24 h after (+) challenge with LPS. Tissues were collected 15 days after challenge with LPS from animals 4 and 5. Animals 3 and 8 were not injected with LPS. Animal 8, from which the axial organ was dissected, had been recently shipped and was assumed to have an activated immune system. The control gene, *SpL8*, is the sea urchin homologue to human *L8* that encodes a ribosomal protein (see Multerer and Smith, 2004) and is constitutively expressed in all tissues. A, B and C denote three statistically different levels of *SpTie1/2* gene expression ($P < 0.05$) from high (A) to low (C) based on ANOVA analysis. The asterisks indicate a significant increase in *SpTie1/2* gene expression in axial organ after challenge ($P < 0.05$). Additional animals were used in preliminary analyses by RT-PCR that showed similar results (data not shown); coelomocyte ($n = 6$), esophagus ($n = 1$), gonad ($n = 1$), gut ($n = 2$), pharynx ($n = 2$). na, did not require LPS challenge for immune activation.

Expression of *SpTie1/2* was also investigated during development of sea urchin embryos from unfertilized egg to pluteus (51.5 h post-fertilization (hpf)). Results from qPCR analysis showed that expression was very low during early development and became detectable at 27 hpf (early gastrula) (Fig. 5). Expression peaked at 36 hpf (late gastrula) and continued to be expressed, but at slightly lower levels until the pluteus stage. The onset of expression coincided with the differentiation of secondary mesenchyme cells into blastocoelar cells, the immune cells in the sea urchin embryo (Smith et al., 2006; Hibino et al., 2006; Tamboline and Burke, 1992; Silva,

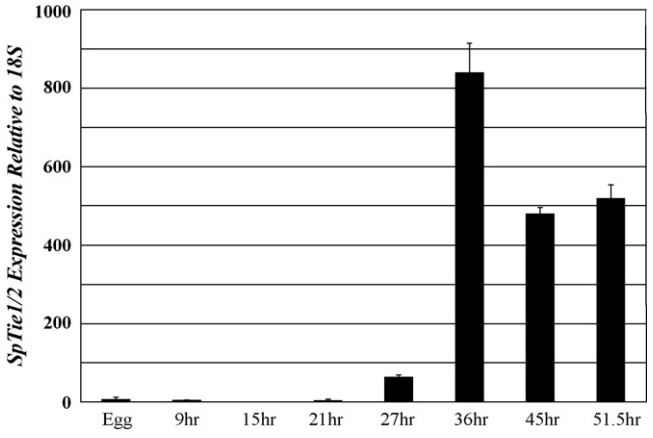

Fig. 5. Embryonic expression of *SpTie1/2* peaks at late gastrula. Total RNA samples were collected from *S. purpuratus* eggs and at 6 h intervals including 9 hpf (cleavage stage); 15 hpf (early blastula); 21 hpf (mesenchyme blastula); 27 hpf (early gastrula); 36 hpf (late gastrula); 45 hpf (early prism); 51.5 hpf (prism). Following reverse transcription, *SpTie1/2* transcripts are detected by qPCR and normalized to 18S RNA, which serves as the loading control.

2000; Furukawa et al., 2009) and suggested a potential role for SpTie1/2 protein in the sea urchin larvae analogous to that of Tie2 in mouse hematopoiesis (Takakura et al., 1998).

3.6. Phylogenetic relationships among Tie homologues

When the *SpTie1/2* gene model was first identified in the sea urchin genome it was reported to encode proteins similar to both vertebrate Tie1 and Tie2 proteins, and therefore was called *SpTie1/2* (Sodergren et al., 2006b; Hibino et al., 2006). To illustrate the phylogenetic relationships among many of the Tie sequences that have become available more recently, Tie-like sequences were collected from amphioxus, *Branchiostoma floridae*, a solitary tunicate, *Ciona intestinalis*, Hydra, *Hydra magnipapillata*, the starlet anemone, *Nematostella vectensis*, honey bee, *Apis mellifera* and mosquito, *Anopheles gambiae*. Several fibroblast growth factor receptors (FGFRs) were added to the analysis, and in some cases were used as the outgroup because they were consistently present in BLAST results as the most closely allied sequence to the Tie family. The tyrosine kinase domain was selected for phylogenetic analysis because it was most highly conserved and most uniform in size among the various domains, which enabled a high quality alignment and robust phylogenetic trees. Several different phylogenetic approaches were used to generate trees and all produced very similar results with respect to the relationship of the SpTie1/2 protein to the human Tie1 and Tie2 sequences (a representative tree is shown in Fig. 6). The vertebrate Tie sequences fell into two clades, Tie1 and Tie2/TEK with SpTie1/2 positioned at the base of these two clades along with *B. floridae* Tie1 and *B. floridae* GFR. Most of the sequences from the invertebrates clustered with the FGFR sequences including the *B. floridae* GFR and Tie1 sequences suggesting that the *B. floridae* Tie1 sequence may not be a Tie homologue. Overall, the SpTie1/2 sequence appears equally similar to vertebrate Tie1 and Tie2/TEK in agreement with a preliminary phylogenetic tree of RTKs identified from the sea urchin genome (Lapraz et al., 2006).

Similar analyses were performed for the Ig-Tie2_1 domain and the second Ig domain at the N terminal end of the proteins (see Fig. 2), which, in mammalian Tie proteins, are involved with ligand binding based on crystal structure (Barton et al., 2006). The resulting trees showed that each SpTie1/2 Ig domain clustered at the base of the Tie1 and Tie2/TEK clades for the respective Ig domain (data not shown). The phylogenetic position of SpTie1/2 at the base of the Tie1 and Tie2 clades in analyses for both the Ig and tyrosine kinase domains suggested that *SpTie1/2* may be descended from a single ancestral *Tie* gene that was present in an ancestral deuterostome prior to duplication in the chordates. The phylogenetic results also suggested that there is only a single Tie homologue in *B. floridae*, and that the duplication of the *Tie* genes occurred in the higher chordates.

3.7. A putative ligand for SpTie1/2

The ligand for vertebrate Tie receptors is Ang, which is a secreted glycoprotein of about 55 kD consisting of a signal sequence and a single fibronectin domain (Davis et al., 1996). To determine whether a sea urchin Ang homologue was present in the genome (v2.0), four vertebrate Ang sequences, human Ang1 (NP_001137.2), human Ang4 (NP_001138), *Danio rerio* Ang1 (NP_571888); and *Danio rerio* Ang2 (NP_5711889.1), were used in BLAST searches. The sea urchin fibronectin domains from candidate Ang-like homologues ($n = 30$) were identified using SMART, aligned with the vertebrate Ang fibronectin sequences ($n = 14$), and used in a variety of phylogenetic analyses. The resulting trees consistently showed that the putative sea urchin Ang-like sequences clustered separately from the vertebrate Ang (types 1, 2 and 4) sequences (data not shown). Although many of the deduced fibrinogen domains from the sea urchin gene models had the Ca^{+2} binding motif, the amino acids important for binding angiopoietin to Tie receptors in vertebrates (Barton et al., 2006) were not conserved in the deduced sea urchin proteins (data not shown). Therefore, identification of the gene model that encodes the activating ligand for SpTie1/2 will require functional analyses of the encoded proteins.

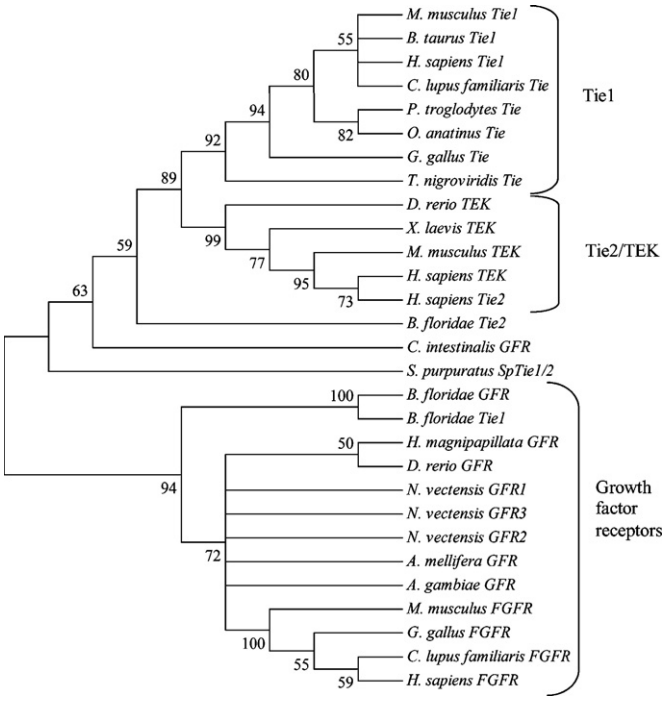

Fig. 6. SpTie1/2 is ancestral to vertebrate Tie/TEK sequences. Phylogenetic analysis of the amino acid sequence for the tyrosine kinase domain are shown as an unrooted 50% consensus tree using minimum evolution (ME) with 1000 bootstrap iterations. Clades for vertebrate Tie1, Tie2/TEK and the growth factor receptors (GFRs) are indicated with brackets. In addition to ME, maximum parsimony, neighbor-joining, and UPGMA trees with 1000 bootstrap iterations were constructed in MEGA4 (Kumar et al., 2004). Maximum likelihood trees were generated with 1000 bootstrap iterations using the randomized accelerated maximum likelihood (RAxML) program (Stamatakis et al., 2008) on the CIPRES (Cyber Infrastructure for Phylogenetic Research) web cluster located at the San Diego Super Computing Center (San Diego, CA). All results were similar to the ME tree shown. The following sequences were obtained from GenBank: SpTie1/2, *Strongylocentrotus purpuratus* Tie1/2 (GQ979611); *M. musculus* Tie1, *Mus musculus* Tie1 (NP_035717.2); *C. lupis familiaris* Tie, *Canis lupus familiaris* Tie (XP_539652.2); *B. taurus* Tie1, *Bos taurus* Tie1 (NP_776390); *H. sapiens* Tie1, *Homo sapiens* Tie1 (NP_005415); *P. troglodytes* Tie, *Pan troglodytes* Tie (XP_524693.2); *O. anatinus* Tie, *Ornithorhynchus anatinus* Tie (XP_001512741.1); *G. gallus Tie*, *Gallus gallus* Tie (XP_422400.2); *T. nigroviridis* Tie, *Tetraodon nigroviridis* Tie (CAG11565.1); *Homo sapiens* Tie2 (BAG58094.1); *D. rerio* TEK, *Danio rerio* TEK (AAI63579.1); *X. laevis* TEK, *Xenopus laevis* TEK (AAK72490.1); *Mus musculus* TEK (CAA50556.1); *Homo sapiens* TEK (BAD92033.1); *C. intestinalis* GFR, *Ciona intestinalis* GFR (XP_002120540.1); *B. floridae* GFR, *Brachiostoma floridae* GFR (XP_002220616.1); *Danio rerio* GFR (AAB63283.1); *H. magnipapillata* GFR, *Hydra magnipapillata* GFR (XP_002157686.1); *N. vectensis* GFR1, *Nematostella vectensis* GFR1 (XP_001641378.1); *Nematostella vectensis* GFR2 (XP_001641254.1); *Nematostella vectensis* GFR3 (ABN70838.1); *A. mellifera* GFR, *Apis mellifera* GFR (XP_396649.3); *A. gambiae* GFR, *Anopheles gambiae* GFR (XP_562866.3); *Mus musculus* FGFR (NP_034337.2); *Gallus gallus* FGFR (NP_990650.1); *Canis lupus familiaris* FGFR (NP_001003336.1); *Homo sapiens* FGFR (NP_075259.4). The following sequences were obtained from (D'Aniello et al., 2008): *B. floridae* Tie1, *Brachiostoma floridae* Tie1; *B. floridae* Tie2.

3.8. Putative downstream signaling proteins

Vertebrate Tie2 proteins have three tyrosines (Y1101, Y1107, and Y1112) located near the C terminus of the cytoplasmic tail that are phosphorylated to enable interaction with SH2 domain proteins such as Grb2 and the p85 subunit of PI3K (Huang et al.,

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HsGrb2            MEAIAKYDFKA-TAD-DELSFKRGDILKVLN-EECDQNWYKAELNGKDGFIPKNYIEM-KP
SpGrb2A-like      ..Yv.LF..SPsKeEQ...A..K.ev...IKR.-.rh.LL.HQGA.Q.LV.e..lkIE-.S
SpGrb2B-like      -..T..h..NG-QeeS-.....kNSi...-----------------------------
SpGFR-bound-like  ---.L...DP--EEEG..C.rkGDIiTlidkPT-kd.wrGTvd.rT.Ml.AP.vkEeEE..
SpGrb3-like       -..D.LR..NGTK.--...P..k..m..i.dRS.HN.D.....RS..e....AK.VRVDN-
                            *                 *                    *  *
HsGrb2            HPWFFGKIPRAKAEEMLSKQRHDGAFLIRESESAP--GDFSLSVKFGNDVQHFKVLRDGAG
SpGrb2A-like      ...yv...S.KV...Y.MSMPS....m..D...N.DS.n.......RdQ.....i.T.L..
SpGrb2B-like      --------T.DG...l.-KNDG.........GT.--..y......VdG.............
SpGFR-bound-like  -....PT.T.KN..LL.MQE.-N....V.R...SE--.LY.....YNeS....Ri.Q.T..
SpGrb3-like       ------------------------------------------e..GG......Q.....
SH2domain         ═══════════════════════════════════════════════════════════

HsGrb2            KYFLWVVKFNSLNELVDYHRSTSVSR-NQQIFLRD----IE---QVPQQP--------TYV
SpGrb2A-like      .........T.i.d.....kDN....-T.e.V.NePCVP..DAN.r..PAMQQSRERLQQ.
SpGrb2B-like      ............q..e...ts....-s.T.y.k.—----------------RKSESIHL.
SpGFR-bound-like  .fh..i...P..dA....y.t...t.E..V------------ST.SVE.WSRNPQRH----
SpGrb3-like       ...y........q..e...aE..----------------------K.ALF------------
SH2 domain        ═══════════════════════

                                                                SH3 domain  ═══

HsGrb2            -QALFDFDPQEDGELGFRRGDFIHVMDNSDPNWWKGACHGQT----GMFPRNYVTPVN
SpGrb2A-like      -K..yn.eSDdAQ..T.kqN.V.TlTGRPHAD..e.EIRAP.TRRT.i..k...KElD
SpGrb2B-like      -L..y..TAG.e...S.kk..R.E.InET.....T.KLVA-.ESQ-.L..ST.TKEiT
SpGFR-bound-like  L...y....e.e...C..k..I.TlI.KPTKd..R.-----.VDGRT..L.AP.V----
SpGrb3-like       -V..Y.YTAe.eL..S.kk..K.K.Fn...Sd..S.VLVA-TGVA-.Q..V..T----
SH3 domain        ═══════════════════════════════════════════════════════════
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Fig. 7. Alignment of predicted amino acid sequence of sea urchin growth factor receptor-bound protein (Grb)-like sequences with human Grb2. Sea urchin sequences were identified using BLASTp with the human Grb sequence to search the *S. purpuratus* genome (v2.1). Dots indicate identities to the human sequence. Amino acids with similar characteristics are indicated by lower case. Asterisks above the alignment denote amino acids that function in the phosphotyrosine binding pocket. The SH2 and SH3 domains are indicated with single and double underlining, respectively. SpGrb2A-like (XP_786023.2 or XP_001175588.1) and SpGrb2B-like (XP_001180827.1 or XP_788430.1) are similar to human Grb2. SpGFR-bound-like (XP_001180717 and XP_001181623) is similar to human Grb2. SpGrb3-like (XP_001180777) is similar to human Grb3.

1995) to initiate signaling. The SpTie1/2 sequence only had a single tyrosine conserved at the C terminus of the cytoplasmic tail (the equivalent Y1101 position; see Fig. 3), which in vertebrates interacts with Grb2 and the p85 subunit of PI3K. Consequently, the sea urchin genome was searched for homologues of Grb2 and p85 using the human Grb2 sequence (CAG29359) and 12 gene models were identified that encode proteins similar to human Grb2. The four gene models with the best matches based on e-values were aligned with human Grb2 (Fig. 7). All of these proteins had SH2 and SH3 domains, and three or four of the conserved basic amino acids expected for a phosphotyrosine binding motif (Inohara et al., 2005). Additional genome searches using human p85 (AK302049) identi-

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HsPI3K 85    PKPPKPMTSAVPNGMKDSSVSLQDAEWYWGDISREEVNDKLRDMPDGTFLVRDASTKMQG  144
             P P K     +  G  D   SL DAEWYWG ISREEVN+KL+DMPDGTFLVRD S +  G
Sp-p85 476   PPPRKSPGDYISTGSTDYPKSLNDAEWYWGSISREEVNEKLKDMPDGTFLVRD-SKQEPG  534

HsPI3K 145   DYTLTLRKGGNNKLIKIYHRDGKYGFSDPLTFNSVVELINHYHHESLAQYNPKLDVKLMY  204
             +YTLTLR                    D +  N + ++I                ++L
Sbjct  535   NYTLTLR-------------------LDDIQDNDIGKVI----------------LQLKD  559

HsPI3K 205   PVSRYQQDQLVKEDNIDAVGKKLQEYHSQYQEKSKEYDRLYEEYTRTSQEIQMKRTAIEA  264
                 YQ              K ++Y   Y+    +Y++L +E       I+ KR A++A
Sbjct  560   KNKEYQD--------------KTKQYDELYE----QYNKLAQE-------IKTKRQALDA  594

HsPI3K 265   FNETIKIFEEQCHTQEQHSKEYIERFRREGNEKEIERIMMNYDKLKSRLGEIHDSKMRLE  324
             FNE + + EEQ             + ++ +  E + ++ NY++L++R  E+ DS+  LE
Sbjct  595   FNEIVTMLEEQLQIHTS--------YHQDCHPAEKQMLLQNYERLENRRQEVKDSRENLE  646

HsPI3K 325   QDLKKQALDNREIDKKMNSIKPDLIQLRKIRDQHLVWLNHKGVRQKRLNVWLGIKNEDAD  384
             + LKK+  ++RE D+ MNS+KP+++ LR +RD ++VWL  KGV+Q ++N WL  ++
Sbjct  647   EQLKKEVEESRECDRTMNSLKPNILDLRNLRDTYVVWLARKGVKQDKINSWLESQDGPG-  705

                                      *                 *
HsPI3K 385   ENYFINEEDENLPHYDEKTWFVEDINRVQAEDLLYGKPDGAFLIRESSKKGCYACSVVA-  443
                    E E   H +E  W + D NR +AE LLYGK  G FLIR + ++  +ACS+VA
Sbjct  706   -------EKEVQYHNNESLWLLPDTNRAKAEQLLYGKAKGTFLIRRTREQIDWACSIVAK  758

                   *  *
HsPI3K 444   -DGEVKHCVIYSTARGYGFAEPYNLYSSLKELVLHYQQTSLVQHNDSLNVRLAYPVHAQM  502
               GEV HC I  T+ GYGFAEPYNLY SL ELVL YQQ +L QHN+ L+  L YPV+
Sbjct  759   DQGEVCHCKINYTSTGYGFAEPYNLYGSLLELVLAYQQINLAQHNEELDTMLEYPVYGPQ  818
```

Fig. 8. BLASTp alignment of two SH2 domains from p85 subunit of human phosphatidylinositol 3-kinase (Hs-PI3K) with that of the sea urchin Sp-p85-like sequence. The sea urchin sequence (Refseq XP_793526.2) was identified by searching the *S. purpuratus* genome (v2.1) with human p85 (BAG63440.1) (*e*-value = $4e^{-70}$). The SH2 domains in the human sequence are underlined. Asterisks denote amino acids that function in the phosphotyrosine binding pocket of the second SH2 domain in the human sequence. Plus signs indicate similar amino acids. Dashes indicate gaps.

Table 2
Polymorphisms within the SpTie1/2 cDNA sequence.

Domain or region	% of ORF	% of SNPs	% of amino acid polymorphisms
Ectodomain: 3 Ig, 4 EGF	38.6	28.1	31.4
Stalk region: 4 FNIII	26.6	54.8	55.6
Tyrosine kinase domain	19.3	10.4	5.0

fied a sea urchin gene model (XP_793526; SPU_015590) encoding a protein with p85 similarity. Alignment of the relevant region of the sea urchin protein against human p85 showed conservation of the four basic amino acids that form the phosphotyrosine binding pocket (Fig. 8). Thus, there were several likely candidates for Grb2-like adapter proteins and a p85 protein encoded in the sea urchin genome that may interact with the cytoplasmic tail of SpTie1/2 to initiate signaling.

4. Discussion

The data presented here confirm and extend the initial reports of a *Tie* homologue in the purple sea urchin (Hibino et al., 2006; Smith et al., 1996; Bradham et al., 2006). The *Sp059* cDNA sequence encodes an integral membrane protein of the RTK family. The characteristics of the tyrosine kinase domain, the types, numbers and arrangements of extracellular domains in the deduced protein, the exon structure of the gene, and the intron positions between the exons encoding in the tyrosine kinase domain all group SpTie1/2 within the Tie family, which is the only known protein family to have this unique combination of extracellular domains (Dumont et al., 1992; Maisonpierre et al., 1993; Partanen et al., 1992; Sato et al., 1993; Jones et al., 2001; Schnurch and Risau, 1993; MacDonald et al., 2006). The tyrosine kinase domain, with a highly conserved catalytic site, nucleotide binding site, and activation loop, is typical of RTK proteins (Kissau et al., 2003) and vertebrate Tie2 in particular (Shewchuk et al., 2000).

Although *SpTie1/2* is a single copy gene, multiple alleles are present in the population (as represented by the various cDNAs that were sequenced) and show 5.5% sequence variation (239 of 4296 nt) that is similar to the 4–5% overall rate of polymorphism estimated for the sea urchin population (Britten et al., 1978). The SNPs and amino acid polymorphisms are not evenly distributed throughout the coding region and are less abundant in functionally important regions of the protein. The conserved regions include the tyrosine kinase domain and the region of the ectodomain including the three Ig domains and an EGF domain that putatively bind the ligand (Table 2). In vertebrate proteins, the Ig and EGF domains in the ectodomain form a compact globular structure that present the second Ig domain outward from the cell surface for binding the Ang proteins (Barton et al., 2006; MacDonald et al., 2006). The stalk of the protein, which is composed of the four FNIII domains, functions to extend the Ang binding site away from the cell surface. The stalk is the most variable region of the sequence with the most SNPs and amino acid polymorphisms. Overall, the distribution of SNPs suggests that the most important functions of SpTie1/2 are carried out by the tyrosine kinase domain and the ligand binding region, which is the same for the vertebrate Tie proteins.

4.1. Exons and structural motifs

The *SpTie1/2* gene differs from the vertebrate *Tie* genes in having more exons (27 vs. 22 for the vertebrate *Tie* genes) that correlate with additional extracellular domains (extra Ig-like, EGF and FNIII domains), plus two exons that are absent from the vertebrate *Tie* genes but that are not associated with a recognizable domain (Figs. S1, 2). Conversely, the number and approximate sizes of exons, and the positions of the exons is highly conserved within the catalytic tyrosine kinase domain among vertebrate *Tie* genes and *SpTie1/2*. This consistency contrasts with the differences that exist among different RTK families in which the number of exons that encode the catalytic region range from five to ten based on SMART analysis of protein sequences. Even the FGFRs, which are the most closely related to Tie family on the basis of similarity searches, are missing one of the introns found in the vertebrate *Tie* genes and in *SpTie1/2* (e.g., D'Aniello et al., 2008). Thus *SpTie1/2* is closely allied with the vertebrate *Tie* genes.

4.2. Possible function of the encoded protein

There are three lines of evidence to consider when speculating on the function of SpTie1/2: (i) the structure of the protein itself, (ii) the function of orthologues in other organisms, and (iii) the pattern of expression within the tissues of both the adult and developing sea urchin. In post-embryonic vertebrates, hematopoiesis in the bone marrow requires periodic division of a self-renewing but relatively quiescent population of stem cells called the long-term repopulating hematopoietic stem cells (LTR-HSCs), which express *Tie2* and require the activity of the functional protein (Hsu et al., 2000). The Tie proteins are not required for fetal hematopoiesis or for the initial production of adult HSCs (Puri and Bernstein, 2003) but are involved in maintaining the LTR-HSCs in a quiescent state that promotes their long-term survival (Arai et al., 2004). Tie1 is not required for the function or survival of HSCs (Partanen et al., 1992; Rodewald and Sato, 1996). Although equivalent cells to LTR-HSCs are not known in echinoderms and the site(s) of coelomocyte proliferation are also unknown, the sea star *Asterias rubens* responds to LPS and concanavalin A with proliferation of coelomocytes, the epithelial lining of the coelomic cavity, the Tiedemann bodies, and, most notably, the axial organ (Holm et al., 2008). The function of the axial organ in the sea urchin is poorly understood, but it is known to contain phagocytic cells, as well as cells that are identical to several classes of coelomocytes; phagocytes, red spherule cells, and vibratile cells (Bachmann et al., 1980). Millott (1966) reported that the axial organ rapidly releases cells into the haemal system in response to injury. Furthermore, ultrastructural analysis suggests that the axial organ functions as an excretory organ and as a site for the destruction of senescent coelomocytes (Bachmann et al., 1980) or foreign cells (Millott, 1966). The presence of coelomocytes in the axial organ, whether it is their proliferative site of origin or whether they are sequestered there for recycling may be the underlying basis for elevated *SpTie1/2* expression. In embryos, it is noteworthy that *SpTie1/2* expression correlates with ingression and differentiation of secondary mesenchyme cells into blastocoelar cells (Kinnander and Gustafson, 1960; Hardin and McClay, 1990) and pigment cells (Gustafson and Wolpert, 1967; Gibson and Burke, 1985; Ruffins and Ettensohn, 1993), which are competent to carry out immune functions shortly after ingression (Smith et al., 2006; Hibino et al., 2006; Silva, 2000; Furukawa et al., 2009). Results from both adult and embryonic gene expression studies suggests that *SpTie1/2* may be involved in immune cell proliferation.

The function of the vertebrate Tie proteins in angiogenesis has been studied extensively because of the importance of this process in tumor formation and tissue repair (reviewed by Martin et al., 2008; Peters et al., 2004). There is good evidence that endothelial and hematopoietic cells develop from a single type of progenitor cell (Huang et al., 1999; reviewed by Schatteman et al., 2006), and Tie proteins are expressed in the endothelial cells of developing blood vessels (Dumont et al., 1992, 1994a,b; Korhonen et al., 1992) and in hematopoietic cells of the fetal liver (Hsu et al., 2000) and bone marrow (Batard et al., 1996; Hashiyama et al., 1996). Neither Tie1 nor Tie2 are required for initial formation of blood vessels in the vertebrate embryo (vasculogenesis), but both are required for

angiogenesis in the adult (Puri et al., 1999), including Tie2 involvement in blood vessel sprouting (Jones et al., 2003; Kim et al., 2000; Takakura et al., 2000). Thus, the roles of the vertebrate Tie proteins in angiogenesis, are to influence the interaction of the endothelium with the underlying connective tissue (Suri et al., 1996), i.e., to regulate the associations of existing cells rather than to regulate cell proliferation. Although sea urchins do not have blood vessels, they do have tubes that function in the water vascular system, which use water hydraulics to move and extend the tube feet for locomotion. Although the water vascular system is a permanent system in the adult, we speculate that the *SpTie1/2* gene may be expressed during late development (a time point that was not evaluated) and function in remodeling the tubes of the water vascular system during metamorphosis. The adult rudiment grows in the larva and upon metamorphosis, everts to form a juvenile sea urchin with five tube feet (Smith et al., 2008). The water vascular system begins to develop in the adult rudiment and likely undergoes remodeling, reorganization and/or expansion during metamorphosis resulting in a pentamerous, sessile, juvenile sea urchin. If *SpTie1/2* plays a role in tubule remodeling that is similar to the vertebrate Tie protein functions in blood vessel formation, the tubules of the water vascular system are a possible candidate. Future work on the developing water vascular system in the adult rudiment and the newly metamorphosed juvenile sea urchin will be required to demonstrate a correlation with *SpTie1/2* expression. Results from both adult and embryonic gene expression studies suggests that *SpTie1/2* may be involved in immune cell survival and/or proliferation based on expression in the axial organ.

4.3. Possible downstream adaptor proteins

Upon ligand binding, the Tie proteins initiate signaling cascades by phosphorylating the cytoplasmic tail so that associated adaptor proteins can bind. There are three tyrosines (Y1101, Y1107, and Y1112) located near the C terminus of the mouse Tie2 protein that, when phosphorylated, enable interaction with adaptor proteins with SH2 domains (Huang et al., 1995). The first tyrosine, Y1101, binds to the SH2 adaptor proteins Grb2 and Grb7 (Jones et al., 1999), while the third tyrosine, Y1112, binds SHP2 adaptors (Peters et al., 2004; Huang et al., 1995). Phosphorylation of Y1101 also promotes binding to the p85 subunit of PI3K (Kontos et al., 1998), which activates the PI3K/Akt pathway (Kim et al., 2000; Jones et al., 1999) promoting cell survival and enhanced cell migration (Peters et al., 2004; Jones et al., 1999; Kontos et al., 1998). Binding of Grb2 can activate the mitogen-activated protein kinase (MAPK) pathway leading to morphogenetic changes in vertebrate endothelial cells (Peters et al., 2004). Vertebrate Tie proteins also interact with the p85 subunit of PI3K, and the activation of Tie2 leads to an inhibition of apoptosis and an increase in cell survival, as well as changes in mobility through effects on the cytoskeleton (Kontos et al., 1998). The equivalent Y1101 position at the C terminus of the SpTie1/2 cytoplasmic tail is the only tyrosine in a conserved position for phosphorylation. Because SpTie1/2 only has one of the three possible functional tyrosines, it may have restricted activities compared to the vertebrate homologues. Phosphorylated Y1101 in SpTie1/2 may bind the homologues of Grb2 and/or p85 to act in a manner analogous to vertebrate Tie2. This infers that SpTie1/2 function in coelomocytes may be involved with the control of the cytoskeletal transformations and changes in adhesion or mobility that have been observed for coelomocytes in contact with foreign substances or cells (Edds, 1985). Because SpTie1/2 only has one of the three possible functional tyrosines, it may have restricted activities compared to the vertebrate homologues.

Unlike Tie2, vertebrate Tie1 has no known ligands, although its activity is influenced by Ang analogues (Saharinen et al., 2005). Tie1 is postulated to regulate the activity of Tie2 by binding to its extracellular domain and interfering with activation by Ang1 (Marron et al., 2007, 2000). Although the two vertebrate Tie proteins are known to interact, the nature of this interaction is still incompletely understood. Tie1 may function in angiogenesis as an agonist to Tie2 activation. It is worth noting that Tie1 from several vertebrates has the consensus sequence YVNM at the binding site for p85 (Y1100/1101), whereas the Tie2 proteins have the sequence YVNT (Fig. 3). This would indicate that phosphorylated Tie1 should bind to p85 more strongly than Tie2 and might activate the PI3K/Akt pathway. The equivalent putative Ang binding site in SpTie1/2 is YVNL, which would seem to be more like the Tie1 sequence because of the hydrophobic amino acid in the fourth position. However, because sea urchin *SpTie1/2* appears to be a single copy gene, the role of SpTie1/2 may be more similar to the vertebrate Tie2 and perhaps function in hematopoiesis of coelomocytes in the axial organ and the differentiation of blastocoelar and pigment cells during early embryonic development. The hematopoietic role might be ancestral, while angiogenesis function may have evolved after Tie gene duplication in vertebrates.

We have confirmed the expression of the *SpTie1/2* gene in the coelomocytes and have identified expression in the axial organ and in the gastrula and post-gastrulating embryo. Our evidence indicates that this gene follows the evolutionary pattern found in many RTKs, in that sea urchins have a single gene whereas vertebrates have two (or more) paralogues (Lapraz et al., 2006). Based on sequence comparisons, domain organization and combination, intron locations, and gene expression in the adult and embryonic sea urchin, we suggest that SpTie1/2 might be acting to regulate coelomocyte mobility and/or proliferation. Elevated expression in the axial organ suggests that it may be a source of coelomocytes and that further investigations of this organ are warranted.

Acknowledgements

We gratefully acknowledge the assistance of Charles Lee, David Lima, Mary Ann McYat, Keri Multerer, Rebecca Easley, Katherine Buckley, Sara Kussman and Daniel Sturgill who provided valuable assistance in the lab. Dr. Dimitar Dimitrov helped with the phylogenetic analysis and Dr. Robert Wallace assisted with some of the graphics. This work was supported by funding from NSF (MCB 0077970) to LCS, an NSF ROA supplement (MCB 0114772) and a sabbatical and Faculty Development grant from Ripon College to MES, the Canadian Institutes for Health Research (MOP74667) and the National Science and Engineering Research Council of Canada (NSERC 458115/211598) to JPR, a Merck Corporation summer research grant to JD and a McNair summer research fellowship to DZ.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2010.03.010.

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